Targeting MYC and MYC Target Genes as Therapeutic Strategies in Childhood Medulloblastoma

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von

Giulio Fiaschetti

aus
Italien

Promotionskomitee
Prof. Dr. Martin Pruschy (Vorsitz)
Prof. Nathan W. Luedtke
Prof. Dr. Michael Grotzer (Leitung der Dissertation)
PD Dr. Alexandre Arcaro

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Giulio Fiaschetti

To my Grandfather Leonardo

List of abbreviations (in alphabetic order):

APC: adenomatous polyposis complex
bFGF: basic fibroblast growth factor
BMP: Bone morphogenetic protein
c-Met: mesenchymal epithelial transition factor
CGH: comparative genomic hybridization
CNS: central nervous system
CSF: cerebrospinal fluid
CSRT: craniospinal radiotherapy
CTD: C-terminal domain
DKO: double knock-out
EGL: external germinal layer
ErbB2: erythroblastic leukemia viral oncogene homolog 2
FRZ: Frizzled
FU: Fused (serine/threonine kinase)
GAD1: glutamate decarboxylase 1
GLI: glioma-associated oncogene homolog 1 (family of transcription factors)
GNP: granule neuron precursors
GS-box: glycine/serine-rich region
GSK3: glycogen synthase kinase
HAT: histone acetyl transferases
HDAC: histone deacetylases
HGF: hepatocyte growth factor
HLHz: helix-loop-helix zipper domain
i17q: isochromosome 17q
ICP: increased intracranial pressure
IGL: internal granule layer
LCA: large-cell/anaplastic
LEF1/TCF: lymphoid enhancer binding factor 1/T cell-specific factor
MAPK: mitogen-activated protein kinase
MB: medulloblastoma
MBEN: medulloblastoma with extensive nodularity
MRI: magnetic resonance imaging

NCI: Notch intracellular domain

NSC: neural stem cells

OTX2: orthodenticle homeobox 2

PDGF: platelet-derived growth factor

PI3K: Phosphatidylinositol 3-kinase

PNET: primitive neuroectodermal tumor

PTCH: Patched (sonic hedgehog receptor)

qRT-PCR: quantitative real-time PCR

RUNX2: runt-related transcription factor 2

RTK: receptor tyrosine kinase

SHH: Sonic hedgehog

siRNA: small-interfering RNA

SMO: Smoothened

SUFU: protein suppressor of Fused

TAD: transactivation domain

TGF-β: Transforming growth factor beta

TRRAP: transformation/transcription domain-associated protein

TrkC: Neurotrophin-3 receptor

WNT: Wingless

WHO: World Health Organization

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Summary

MYC and its target genes have become attractive targets for cancer therapy, due to the correlations of the oncogene with high grade malignancy and poor prognosis in different types of tumors. My research investigations focused on novel strategies to target the oncogene *MYC* and/or its target genes, in the context of MYC-overexpressing embryonal tumors (ET), and in particular in medulloblastoma (MB).

Searching for molecules potentially useful as MYC inhibitors, we studied the quassinoids, a group of compounds known in medicine for their wide spectrum of activities. Quassinoids showed anti-cancer properties including cytotoxicity, protein synthesis inhibition, induction of apoptosis, and the capacity to down-regulate the oncogene MYC. Therefore, considering that future research into chemical modifications is likely to generate more active and less toxic derivatives of natural quassinoids, this family represents a source of promising small molecules targeting key pro-survival signaling pathways necessary for the life of a cancer cell.

In this context, we investigated the wide range of biological activities exerted by a semi-synthetic quassinoid analogue named NBT-272. This compound induces *in vitro* and *in vivo* down-regulation of MYC and a complex pattern of cellular responses, functionally related to interference with key cell survival pathways playing a role in the pathogenesis of several ETs. Our findings increase the present level of understanding of the anti-tumor mechanisms of action of the quassinoids, and in particular of the derivative NBT-272.

Our studies also focused on a different approach for targeting MYC. S2T1-6OTD is a molecule able to suppress both mRNA and protein expression of MYC, acting through the stabilization of the quadruplex structure in the promoter sequence of the oncogene. We studied the S2T1-6OTD–mediated cellular effects in a representative set of childhood MB and atypical teratoid/rhabdoid tumor cells. S2T1-6OTD reduces the activity of MYC in a cell-type specific manner, together with a negative effect on cell viability and proliferation of several tumor cell lines. Altogether, our results suggest that S2T1-6OTD may represent a potential effective and innovative therapeutic strategy for childhood brain tumors.

Although the MYC functions during normal development and oncogenesis in various systems have been extensively investigated, the transcriptional targets mediating MYC effects in MB are still elusive. A better knowledge about the MYC's effector genes involved in MB onset

and progression is of great relevance in order to find novel and suitable therapeutic strategies. Our experimental approach to investigate MYC-regulated genes consisted of using a model of MB-derived cells and profiling them by cDNA microarray upon genetic manipulation aiming at either over-expressing or down-regulating *MYC*. We defined a list of 209 candidates with potential relevance to MYC-dependent cellular responses in MB. The gene expression analysis we performed brought to our attention components of the bone morphogenetic protein (BMP) signaling pathway, which plays a crucial role during the development of the cerebellum. Our investigation shows for the first time the existence of a functional link between the over-expression of *MYC* and abnormal regulation of the BMP pathway in a model of MB.

Our results provide evidence of the induction of BMP7 as a MYC-dependent mechanism and indicate this growth factor as a direct target of MYC and as potential effector protein during MYC-driven neoplastic transformation of MB precursor cells. The functional relevance of the BMP-dependent signaling pathway in MB was evaluated by targeting the BMP pathway via specific siRNA-mediated silencing of *BMP7* and employing a small-molecule inhibitor able to prevent the activation of BMP-downstream elements. This new information not only contributes to a better understanding of the pro-survival functions of MYC during MB development, but it also suggests a rationale for targeting BMP pathways as a therapeutic intervention strategy for MB patients with MYC amplification/overexpression.

Taken together, the studies presented in this dissertation have investigated novel strategies to target the oncogene MYC and its target genes in ETs. Further validation, as well as translation to clinical studies, can, in the future, shed more light on the biology of these pediatric tumors and contribute to an improvement of therapies.

Zusammenfassung

Das Onkogen MYC und die dadurch regulierten Gene stehen seit geraumer Zeit im Forschungsinteresse für die Entwicklung neuer Krebstherapien, da MYC mit dem Auftreten hochgradig maligner Tumore und schlechter Prognose in unterschiedlichen Krebsarten korreliert. Im Mittelpunkt meiner Forschung stand die Untersuchung neuer „Targeting-Strategien“ für das Onkogen MYC in embryonalen Tumoren inklusive Medulloblastom, dem häufigsten kindlichen malignen Hirntumor.

Auf der Suche nach potenziellen MYC-Inhibitoren stiessen wir auf die Gruppe der Quassinoide, die für ihr breites Wirkungsspektrum inklusive Zytotoxizität, Hemmung der Proteinbiosynthese, Induzierung von Apoptose und Herunterregulierung des Onkogens MYC bekannt sind. In diesem Kontext untersuchten wir die biologischen Aktivitäten des weniger toxischen semi-synthetischen Quassinoid-Analogs NBT-272. Dieses Molekül induziert Herunterregulierung von MYC *in vitro* und *in vivo* und ein komplexes Muster zellulärer Antwortmechanismen. Die Ergebnisse unserer Arbeit erweitern das momentan vorhandene Wissen über die Wirkungsweise der Quassinoide und charakterisieren das Krebshemmende NBT-272.

Als weitere„Targeting-Strategie“ für das Onkogen MYC untersuchten wir das Molekül S2T1-6OTD, welches sowohl die mRNA- als auch die Protein-Expression von MYC supprimiert, indem es die Quadruplex-Struktur in der Promotor-Sequenz des Onkogens stabilisiert. Unser Interesse galt den S2T1-6OTD-induzierten zellulären Effekten in einem repräsentativen Set von pädiatrischen Medulloblastom- und Atypisch Teratoid/Rhabdoid Tumorzellen. S2T1-6OTD senkte die Aktivität von MYC abhängig vom Zelltyp gemeinsam mit einem negativen Effekt auf die Zellvitalität und -Proliferation.

Obwohl die Funktionen von MYC sowohl in der normalen Entwicklung als auch in onkogenen Prozessen bereits in verschiedenen Modellsystemen intensiv untersucht wurden, sind die transkriptionellen Targets, welche die Effekte der Überexpression von MYC im Medulloblastom charakterisieren, weitgehend unbekannt. Um Medulloblastom-spezifische MYC-regulierte Gene zu finden, wurden MB Zellen mit einer MYC –Überexpression und MB Zellen, in denen MYC herunterreguliert war, einer cDNA Microarray Analyse unterzogen. Aus diesem Versuch konnten wir eine Liste von 209 Genen mit potentieller MYC-abhängigen Relevanz erstellen, die u. a. Gene des „Bone Morphogenetic Protein“ (BMP) -Signalwegs beinhaltete. Dieser Signalweg spielt eine wichtige Rolle in der

Entwicklung des Kleinhirns. Unsere Forschung zeigte zum ersten Mal eine Verbindung zwischen der Überexpression von MYC und einer Dysregulierung des BMP-Signalwegs im Medulloblastom. Unsere Ergebnisse lassen darauf schliessen, dass der Wachstumsfaktor BMP7 MYC-abhängig induziert wird und es sich hierbei um ein direktes MYC-Target handelt. Um die funktionelle Relevanz des BMP-abhängigen Signalwegs im Medulloblastom zu beurteilen, wurde der Signalweg durch das Ausschalten von BMP7 mithilfe von siRNAs herunterreguliert oder durch einen Inhibitor gehemmt. Zusammengefasst führen diese neuen Informationen zu einem besseren Verständnis der „Pro-Survival“ Funktionen von MYC während der Medulloblastom-Entstehung. Zudem bilden sie die Grundlage, um BMP-Signalwege in Patienten mit Medulloblastom zu hemmen, die eine MYC-Amplifikation oder -Überexpression aufweisen.

In der vorliegenden Dissertation wurden neue “Targeting-Strategien” für das Onkogen MYC in embryonalen Tumoren untersucht. Dadurch wurde das Verständnis der Biologie dieser Tumoren verbessert und es wurden Grundlagen geschaffen für die Entwicklung von zukünftigen Therapiemöglichkeiten.

Introduction

Embryonal tumors of the central nervous system

Central nervous system (CNS) tumors are highly malignant and characterized by poorly differentiated neuroepithelial cells with the potential capacity for divergent differentiation [1]. Brain tumors are the most common form of solid tumors in childhood and are second in incidence only to leukemia among all cancers in patients younger than 15 years of age [2, 3]. The rate is inversely proportional to age, with 3.5 to 4 cases every 100,000 children younger than 5 years compared to 2.5 cases every 100,000 children between 10 and 15 years old. The occurrence of brain tumors is higher in boys than in girls, with a ratio of 55:45 [4]. Approximately 50% of all childhood brain tumors arise in the posterior fossa. In this region of the brain, the most common tumors are medulloblastoma (MB), cerebellar astrocytoma, ependymoma, and brain stem glioma [5]. The generic term "primitive neuroectodermal tumor" (PNET) has been historically used to define embryonal tumors (ET), regardless of their site of origin in the CNS [6]. PNET are high-grade undifferentiated neuroepithelial tumors with a primitive embryonal cellular morphology [7, 8]. However, growing evidences suggest that ET are not a uniform group, but rather a heterogeneous group of neoplasms identified by distinct tumor location, different histopathologic origin, and various patterns of differentiation [7]. In 2007, the World Health Organization (WHO) classified the tumors of the CNS in the following subgroups: MB, CNS PNET (medulloepithelioma, ependymoblastoma, CNS neuroblastoma and CNS ganglioneuroblastoma) and atypical teratoid/rhabdoid tumor [9].

Medulloblastoma

MB is the most common malignant brain tumor in children [8], accounting for approximately 20% of primary CNS neoplasms and 40% of all posterior fossa tumors (Fig.1). It is a highly invasive embryonal neuroepithelial tumor that arises in the posterior fossa, usually from the cerebellar vermis in the roof of the IV ventricle. In a minority of cases, particularly in older children, MB arises in a cerebellar hemisphere.

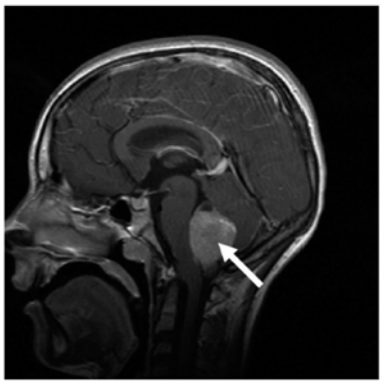

Figure 1. Sagittal gadolinium-enhanced MRI scan. MB tumor indicated by arrow. **(Adapted from [10])**

MB is characterized by an aggressive clinical behavior and a high-risk of leptomeningeal dissemination [11, 12]. The Central Brain Tumor Registry of the United States reported that the incidence of MB in patients up to 19 years old ranges from 0.48 (girls) to 0.75 (boys) every 100,000 patients per year. In the European Union there are approximately 650 new cases per year [10]. MB tumors present two peaks of incidence, i.e. between 3 and 4 years and between 8 and 9 years of age [13]. Although MB can also occur in adults, it accounts for less than 1% of the adult CNS tumors [14].

Multimodal therapy (surgery, radiotherapy and chemotherapy) has improved the overall survival rate, and the expected disease-free five-year survival rate has risen to more than 60%. Using current treatments, approximately 80% of patients without disseminated disease can be cured, although about 30% of patients are still incurable. Moreover, many patients suffer from severe cognitive and neurological dysfunctions. Growth deficiency is observed in 70-80% of patients, thyroid and gonadotropin hormonal deficiency may also occur [13, 15].

Clinical symptoms of MB are ataxia, hydrocephalus, and increased intracranial pressure (ICP), caused by the progressive occlusion of the fourth ventricle. Initial signs of increased ICP are usually subacute, non-specific, and non-localized (i.e. headaches, vomiting, and lethargy). Infants may also be affected by irritability, anorexia, and developmental delay. Later signs are generally the effects of tumor invasion into the surrounding tissues. Tumor infiltration of the brain stem or increased ICP may result in diplopia (the simultaneous perception of two images of a single object), and facial weakness.

Staging and risk stratification

Staging and subsequent patients' stratification are crucial in the management of MB treatment [8]. Molecular markers and histopathological sub-classification do not currently influence the therapeutic strategy and only clinical variables are used in the therapeutic stratification of MB patients [10]. Current staging classification requires analysis of the cerebrospinal fluid (CSF) and magnetic resonance imaging (MRI) of the brain and the entire spine. Assessment of the CSF for disseminated disease is crucial, because up to 30% of

children show disseminated disease at presentation [16]. M_0 staging is assigned if there is no evidence of metastasis, whereas the M_1 stage denotes malignant cells in the CSF. Patients with tumors that are visible on MRI have M_2/M_3 classification. M_4 stage is assigned if there is extraneural spread (e.g. bone and bone marrow), which is very uncommon.

Patients are generally divided into risk-adapted schemes on the basis of age at diagnosis, extent of postoperative residual tumor volume and tumor metastasis [8, 17, 18] (Fig.2). Patients older than 3 years are assigned to the average-risk category (or standard-risk), if they are at stage M_0 with less than, or equal to, 1.5 cm^2 of post-operative residual tumor. Average-risk patients account for 60–70% and, within them, the 5-year survival rate after craniospinal radiotherapy and chemotherapy is currently 70-80% [19]. High-risk patients include those with disseminated disease at diagnosis (stage M_1-M_4) and/or with greater than 1.5 cm^2 tumors as residual disease. The 5-year survival rate for this group is currently 30-60% [20].

A third group are patients younger than 3 years old. This group has the worst prognosis, regardless of M stage and extent of the post-operative residual disease. The 5-year survival rate is approximately 30%, although patients harboring metastasis have even lower survival rate. Poor outcomes in infants can be due to various factors, including increased risk of metastatic disease at presentation, increased rate of subtotal resection, and the impossibility to receive craniospinal radiation therapy [16].

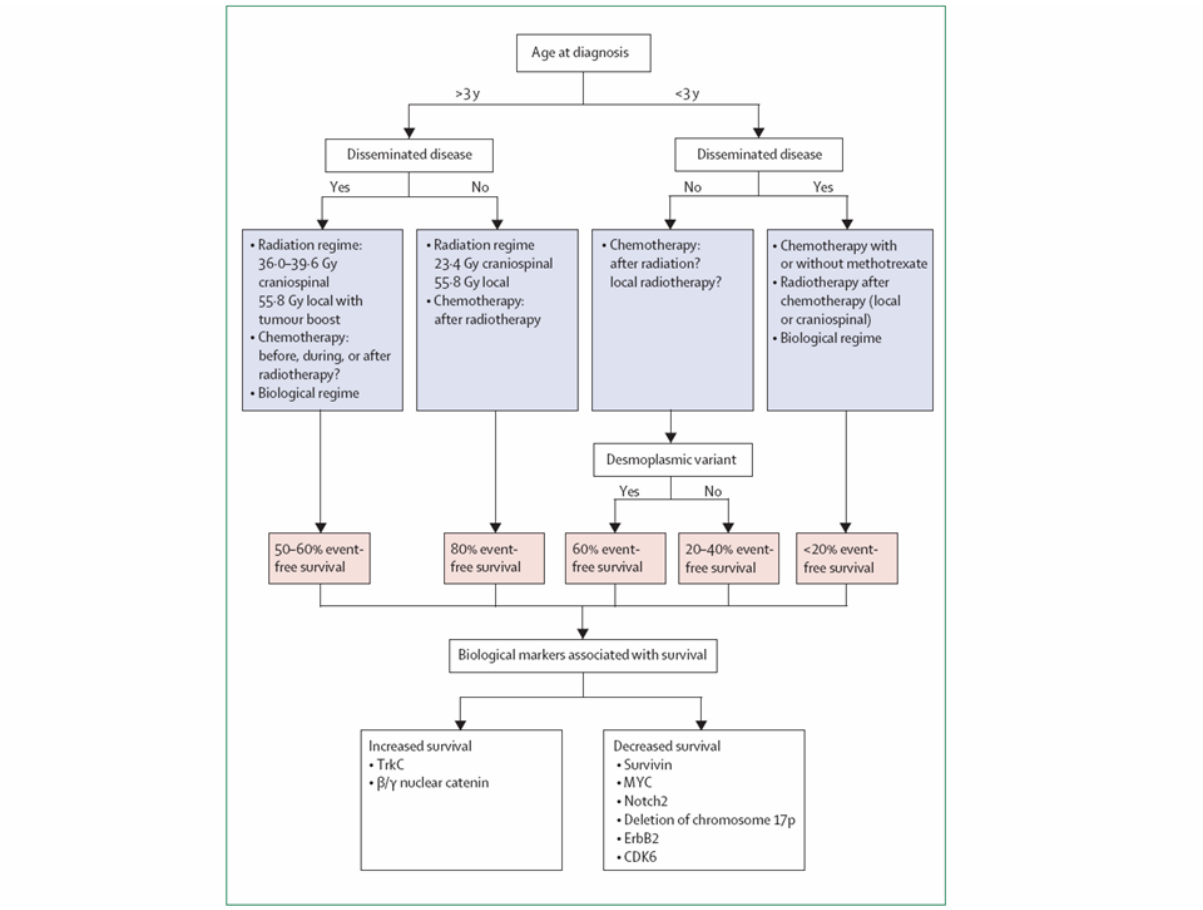

Figure 2. Treatment scheme for MB. A generalised algorithm for the stratifi cation and treatment of MB on the basis of age and risk stratifi cation. **(Adapted from [13])**

Several molecular and histopathological biomarkers have been identified and may allow a more accurate prediction of MB outcome, such as the β-catenin status as a favorable-risk marker, *MYC* gene amplification and large-cell histology as high-risk markers, together with the expression levels of the genes encoding for neurotrophin-3 receptor (TrkC), erythroblastic leukemia viral oncogene homolog 2 (Erbb2), survivin, and p53 [21-25]. These markers may be incorporated into future stratification schemes resulting in better risk-adapted therapies.

Treatment

Despite the recent improvements of survival rates, the delivery of more patients-tailored therapies with intensified treatment of poor-risk diseases and reduced therapy for favorable-risk cases is still one of the major goals in pediatric neuro-oncology. The standard therapeutic approach for MB consists of surgical resection, followed by adjuvant treatment with both postoperative craniospinal radiotherapy (CSRT) and chemotherapy [10].

Surgical resection of MB has led to improved survival in randomized trials, particularly in children with localized disease [17, 26]. One of the most challenging aspects of surgical resection of MB is its vicinity to the brainstem, which has a substantial risk when complete resection is attempted.

Post-surgical treatments in patients older than 3 years include chemotherapy and radiotherapy, with distinct regimens for standard-risk and high-risk patients. The tendency of MB tumors to spread by the CSF led to the early use of CSRT, supplemented with local boost radiotherapy. Previously, conventional radiotherapy protocols for average-risk and high-risk groups required 36 Gy of photon radiation to the craniospinal axis, with a boost to the posterior fossa to a total dose of 54–55.8 Gy. The aim of irradiation of the craniospinal axis is to eliminate possible residual microscopic diseases, not detected by the CSF analysis or by MRI. Due to severe radiotherapy-mediated long-term effects in many MB patients, efforts have been undertaken to reduce the doses of craniospinal irradiation in some patients [19, 27-29]. For high-risk patients with disseminated disease, there is no evidence that the dose or volume of radiotherapy can be reduced [30]. Radiation therapy is delayed or avoided to children younger than 3 years, because of the severe side-effects on the developing brain [7, 8].

MB is a chemosensitive tumor and there is evidence of the benefits of chemotherapy for patients belonging to both standard- and high-risk groups [31]. Chemotherapy can augment, delay, or even prevent the need for radiation treatment, and, after resection, chemotherapy alone can be curative in patients with non-metastatic MB [26]. A range of chemotherapeutic drugs have been used in the treatment of MB and different regimens have shown significant benefits. The most common is a combination of vincristine combined with radiotherapy and CCNU (Lomustine), cisplatin and vincristine, or cyclophosphamide, cisplatin, and vincristine following radiotherapy [19, 32]. After treatment with appropriate doses of craniospinal and local boost radiation and adjuvant chemotherapy, more than 75% of children with average risk MB can be expected to survive 5 years after diagnosis, with the majority being completely cured [13].

Histopathologic and cytogenetic features

MB can be subdivided into the following histological variants: classic, desmoplastic/nodular, large-cell, anaplastic and MB with extensive nodularity (MBEN) [33, 34]. About 70-75% of MBs present a classic phenotype, composed of sheets of packed small round or ellipsoid

cells, occasionally forming neuroectodermal rosettes, with high nuclear-to-cytoplasmatic ratio and frequent mitoses (Fig.3; panel A) [7]. About 15-25% of the tumors are desmoplastic/nodular MBs. This subtype displays a biphasic architecture that consists of regions with dense intercellular reticulin and nodular reticulin-free zones (Fig.3; panel B). Desmoplasia, a pericellular deposition of collagen, occurs in up to 50% of the tumors in very young children, and it is a strong predictor of low risk disease in early childhood [7]. MBEN is a distinctive type of desmoplastic MB and comprises around 5% of all tumors. It occurs mostly in infants and differs from the desmoplastic nodular variant by exhibiting extensive nodularity and advanced neuronal differentiation within the nodules [7, 9]. The large-cell variant of MB accounts for less than 5% of cases and has been associated with poor prognosis [33]. The cells are large and round with a prominent single nucleolus (Fig.3; panel C) [35]. Large-cell MBs contain regions where the round cells give way to densely packed polyhedral cells forming a paving-like pattern. Mitotic and apoptotic indices among these cells are higher than in other MB subtypes [36]. Historically, large-cell and anaplastic MBs have been described as a single subtype of MB, large-cell/anaplastic (LCA), accounting for 10-15% of patients. However, in 2007 the WHO classification of CNS tumors, defined anaplastic and large-cell MBs as two distinct entities [9]. Anaplastic MB accounts for about 10% of cases and represents the most aggressive subtype (Fig.3; panel D). It is characterized by increased nuclear size, marked nuclear pleomorphism and a high mitotic count. Anaplastic MB is associated with high degree genomic instability, and deregulated expression of the oncogene *c-MYC*, thus leading to poor prognosis [7, 37, 38].

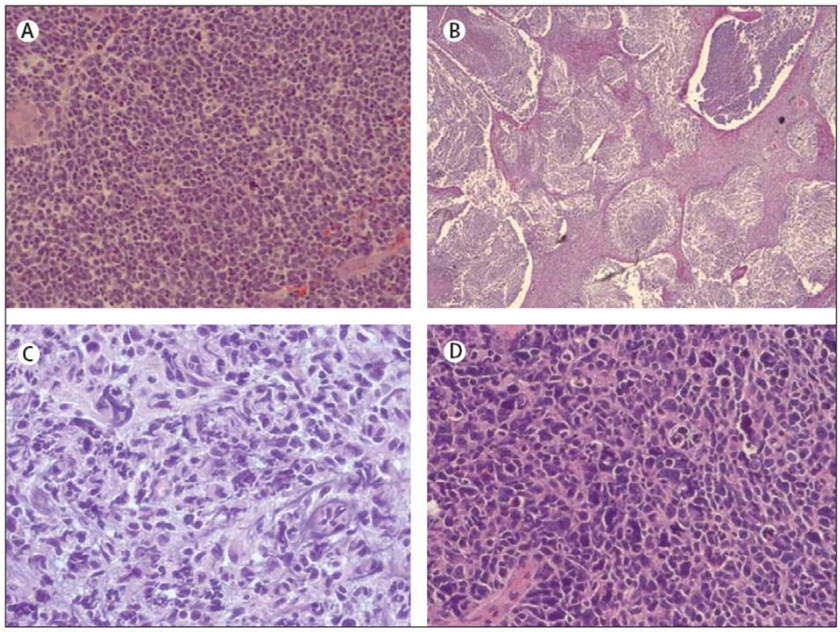

Figure 3. Histological features of MB. A. Classic MB: sheets of small round blue cells with scant cytoplasm. B. Desmoplastic MB: nodular areas of densely packed, reticulin-rich cells. C,D. Large cell (C) and anaplastic (D) MBs are shown. Histological features show prominent and pleomorphic nuclei with large areas of necrosis and high mitotic rate. Cells stained with haematoxylin and eosin. **(Figure adapted from [13])**

Recently, five MB subgroups (A-E) have been defined based on the distinct genetic profiles, specific genetic alterations, pathway signatures, and clinical-pathological features [39]. Subgroup A is characterized by signaling alteration in the wingless (WNT) pathway. More specifically, many genes of this pathway and related target genes have been found over-expressed (e.g. *MYC*, *RUNX2*, and *GAD1*). Mutations in β-catenin were identified in all tumors of the A subgroup, but not in any other MB tumor subtype. The A type is also characterized by exclusive expression, or overexpression, of members of the transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) pathways (i.e. *BMP4*, *BMP7*, *BAMBI*, *SMAD3*, *TGFβI*, and *INHBA*). Cluster B is marked by the sonic hedgehog (SHH) signaling, mutations of *PTCH1* (sonic hedgehog receptor, Patched), and low levels of the oncogene *OTX2* (orthodenticle homeobox 2). Clusters A and B share increased expression of genes involved in protein biosynthesis, cell-cycle, Notch and platelet-derived growth factor (PDGF) pathways. Cluster C and D are marked by an increased expression of neuronal differentiation genes, whereas clusters D and E by expression of photoreceptor genes [39, 40].

Comparative genomic hybridization (CGH) analysis identified several subgroup-specific chromosomal aberrations. Monosomy of chromosome 6 occurs only in type A tumors, whereas loss of 9q mostly occurs in type B tumors. Aberrations of chromosome 17 are

associated with type C and D tumors. Loss of the inactivated X-chromosome is highly specific for female cases of type C, D, and E tumors.

Metastatic disease at diagnosis is significantly associated with the subtype E and, to a lower extent, to subtypes C and D. Patients below 3 years of age are classified as type B, D, or E tumor. The type B includes most desmoplastic cases [39, 40].

Abnormalities of the chromosome 17 are the most common chromosomal aberrations in MB. Isochromosome 17q (i17q) is observed in about 35% of classic and LCA, and 12% of nodular/desmoplastic [34, 41]. The presence of i17q in tumors is associated with a poor clinical outcome, suggesting that it may contribute to the development of aggressive variants of MB [7]. Isolated loss of 17p is observed in an additional ~20% of cases. Gain of chromosome 7 affects approximately 40% of cases [42]. Monosomy of chromosome 6 frequently occurs in tumors with favorable prognosis, mainly classic subtype [43]. Extensive non-random losses of either chromosomes 8, 9, 10q, 11, or 16q are observed in approximately 30% of cases [42, 44].

Alterations in gene expression have been described in different MB subsets [41]. *MYCN* (at 2p24) and *c-MYC* (at 8q24) genes are the most commonly amplified loci, which have been documented across multiple studies. Each event occurs in 5 to 15% of MB cases, and is associated with adverse clinical prognosis [21, 33, 34]. A recent study showed that MB cells transduced with *c-MYC* adopt a severely anaplastic phenotype when grown as xenografts in nude mice, suggesting a causative relationship between *c-MYC* expression and the LCA phenotype [37]. Additional gene amplifications affecting other genetic loci were identified in MB, although with lower frequency, e.g. amplification of *OTX2*, *Notch2*, *TERT*, *MYCL1*, *PDGFRA*, *KIT*, *MYB*, and *CDK6* [45-48].

Origins of MB: disorders of cerebellar development

Normal cerebellum development

The cerebellum derives from the alar (dorsal) plate of the neural tube and resides in the metencephalon, positioned along the anterior/posterior axis of the neural tube [49]. The cellular architecture of the cerebellum derives from two distinct germinal zones: the ventricular zone, a thin layer lining the fourth ventricle and the inner region of the cerebellar anlage, and the external granule layer (EGL), in the vicinity of the dorsal portion of the neural tube, a region known as rhombic lip (Fig.4) [50].

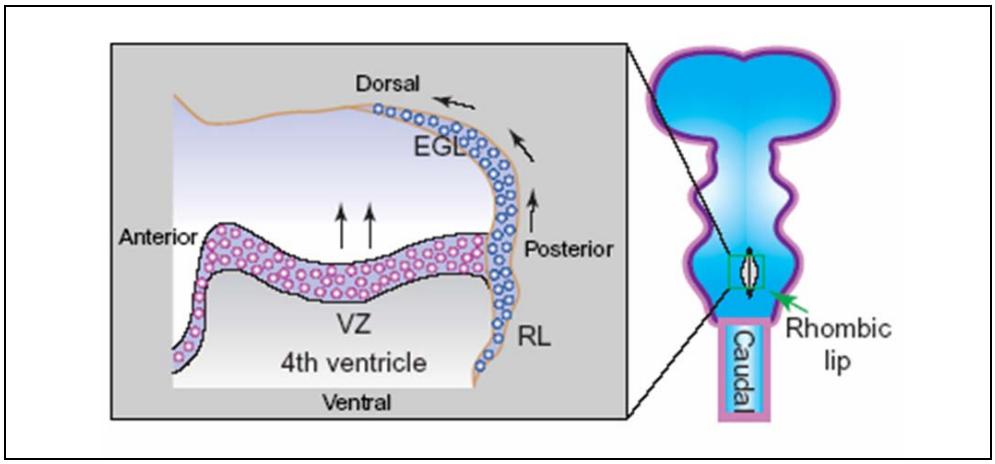

Figure 4. Schematic representation of embryonal cerebellar development. Progenitor cells located in the ventricular zone (vz, primary germinal zone) migrate radially to give rise to Purkinje cells. Progenitor cells located in the rhombic lip (RL) migrate dorsally to populate the external granular layer (EGL, secondary germinal zone). **(Adapted from [51])**

Distinct populations of neural precursor cells are present in the cerebellum: GABAergic neurons, glutamatergic projection neurons and granule neuron precursor (GNP) cells. The precursor cells of the GABAergic neurons, including Purkinje cells and interneurons, are located within the primary germinal zone in the roof of the IV ventricle; glutamatergic projection neurons of the deep cerebellar nuclei and GNP cells derive from the rhombic lip [52-54]. Recently, an additional stem cell population has been described. These cells express neural stem cell markers (CD133 and Nestin) and display the potential to generate astrocytes, oligodendrocytes, and neurons [55].

The distinct precursor cell populations show different properties and require different combinations of intrinsic and extrinsic signaling molecules to function properly. The signaling systems regulating the precursor cells of GABAergic neurons and glutamatergic

projection neurons are still elusive. In contrast, the signals regulating GNP cells have been extensively investigated.

During development, specification, proliferation and differentiation of GNPs are physiologically regulated by a complex network of signaling pathways, including SHH, WNT, BMP, and Notch cascades (Fig.5) [56, 57]. In the rhombic lip, a subgroup of BMPs (BMP6, BMP7, and Gdf7) are secreted by cells in, and adjacent to, the roof plate and provide a signal for specification of GNP cells [54], while SHH signals secreted from Purkinje cells drive their proliferation [58-60]. GNPs first migrate over the cerebellar surface to form the EGL and later migrate inwards, through the Purkinje layer, to form the internal granular layer (IGL) [61]. Prior to form the IGL, a distinct subgroup of BMP cytokines (BMP2 and BMP4) suppresses the proliferative response mediated by SHH and promotes cell-cycle exit and differentiation of GNP cells [62]. The Notch and WNT pathways also coordinate proliferation and differentiation of GNP cells by regulating the neurogenic transcription factor activity and by maintaining neural progenitor cells [63]. The phosphatidylinositol 3-kinase (PI3K) and basic fibroblast growth factor (bFGF) pathways take also part to the signaling modulation, respectively potentiating and inhibiting the response of GNP cells to SHH.

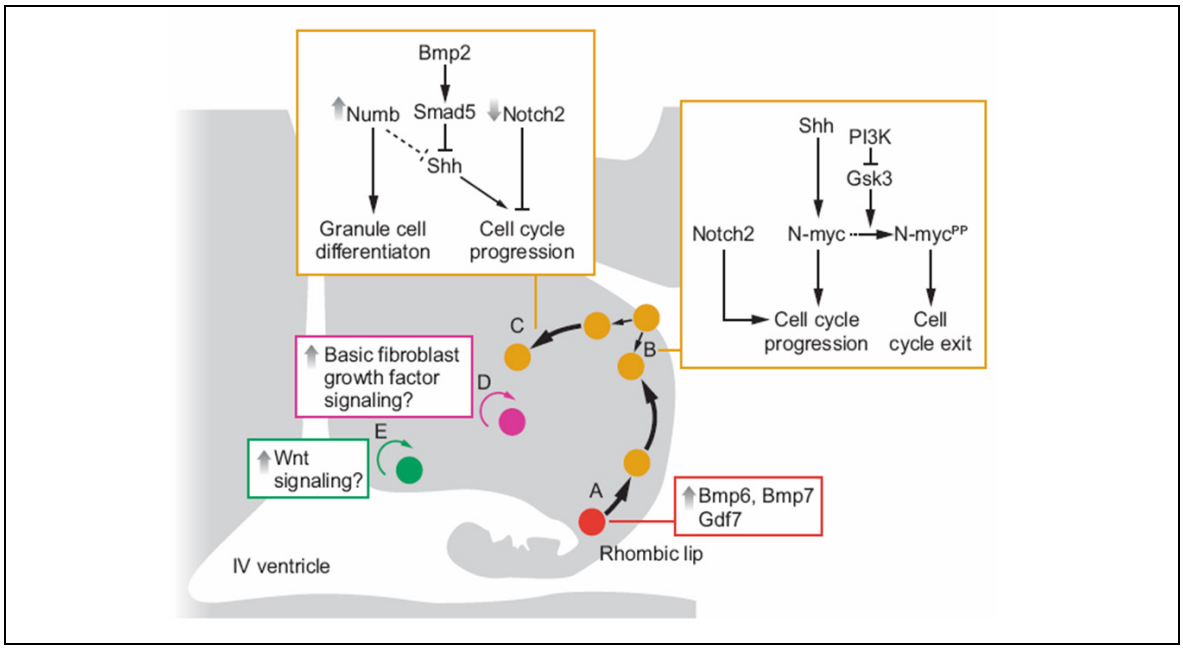

Figure 5. Signaling pathways involved in cerebellar development. (A) Bmp6, Bmp7, and Gdf7 provide a signal for specification of granule cell. (B) GNP cells within the external germinal layer proliferate in response to SHH secreted from Purkinje cells. GNP cells proliferation may be potentiated by PI3K and Notch signaling. (C) BMP2 promotes GNP cycle exit by suppressing the proliferative response mediated by SHH. Increasing Numb expression and decreasing Notch2 expression causes GNP cells to leave the cell-cycle and differentiate into mature granule cells. (D) White-matter stem cells may be sensitive to mitogenic signals mediated by basic fibroblast growth factor. (E) GABAergic precursor cells may depend upon WNT signals. **(Adapted from [7])**

Aberrant signal transduction in medulloblastoma tumorigenesis

MB is associated with alterations of the fine-tuned balance of the described signaling pathways that are physiologically involved in cerebellum development, which is characterized by a complex spatio-temporal regulation of survival, proliferation, and differentiation of multipotent neural progenitor cells, and it is driven by a variety of extracellular signaling molecules, growth factors and cytokines [51, 64]. Even though the precise cells of origin of MB remain still to be fully determined, there is now considerable evidence that tumorigenesis of a subset of tumors can be related to aberrant regulation of the fate of GNP cells [13, 51]. In fact, MBs often arise on the surface of the cerebellum, where the EGL is located, and frequently express markers of GNP cells. Moreover, the extended embryonic and postnatal period of GNP proliferation makes these cells highly susceptible to transforming mutations, which is a prerequisite for neoplastic transformation [13, 65]. The involvement of an altered development of GNP cells in the onset of MB is also supported by studies on patched mutant mice models of MB [64, 66-68].

Beside the subset of MBs arising from aberrant GNP cells in the EGL, Sutter *et al.* recently described the formation of MB tumors from postnatal neural stem cells (NSC) not expressing granule cells markers [69]. NSC were isolated from mice cerebellum and injected subcutaneously in mice, upon inactivation of the tumor suppressor genes *Rb* and *p53*. The resulting tumors were characterized by high expression of *Sox2* and *Sox9* genes, in contrast to GNP-derived MBs which generally show activation of *MYCN*, *GLI* and *PTCH2* [69].

The following sections describe the most commonly aberrant signaling pathways in MB (Fig.6). A particular emphasis will be dedicated to the oncogene MYC and to the BMP pathway, for the relevance they have in development and progression of MB and in the present study.

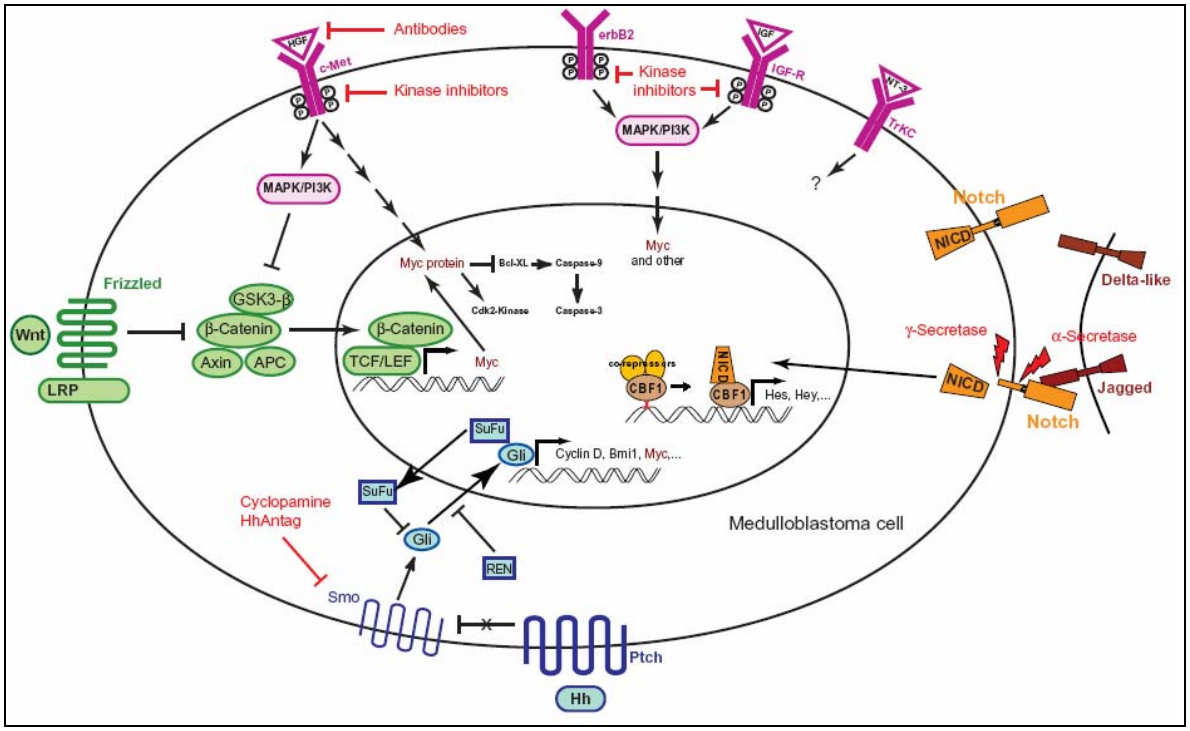

Figure 6. Schematic representation of signaling pathways in MB. Sonic hadgehog pathway in blue: Hh, Sonic hedgehog; Ptch, patched; Smo, smoothened; SuFu, suppressor of fused. **Notch pathway in red/orange:** NICD, Notch intracellular domain; CBF, C promoter-binding factor. **Wingless pathway in green:** Wnt, wingless; LRP, LDL-receptor-related protein; GSK, glycogen synthase kinase; TCF/LEF, T-cell factor/lymphoid enhancer factor; APC, adenomatous polyposis coli. **Receptors tyrosine kinases and IGF signalings in purple:** HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IGF-R, insulin-like growth factor receptor; MAPK, mitogen-activated protein kinase; PI3K, phosphotidylinositol-3-kinase; CDK, cyclin-dependent kinase; P, phosphorylated state. **(Adapted from [64])**

Sonic hedgehog pathway

SHH is a member of a family of evolutionarily conserved proteins. During normal cerebellar development, SHH ligand is secreted by Purkinje neurons and promotes proliferation of GNP cells in the EGL in a concentration-dependent manner [70, 71]. As GNP cells migrate through the Purkinje layer to the IGL, the reduced concentration of SHH, together with the presence of other factors, mediates cell-cycle arrest and differentiation [50, 72].

The SHH-receptor Patched (PTCH) and its associated transmembrane protein Smoothened (SMO) control the response to the SHH signal. PTCH is mainly expressed in EGL precursor cells and, in absence of SHH, it represses the activity of SMO, thereby preventing the transcription of target genes (Fig.7) [73]. The transcriptional response is mediated by the glioma-associated oncogene homolog 1 (GLI) family of transcription factors [74]. In the cytoplasm, GLI forms a protein complex with the serine/threonine kinase Fused (FU) and the suppressor of Fused (SUFU). This complex promotes ubiquitination and degradation of GLI, leading to repression of the pathway. In the presence of SHH, PTCH is blocked and no longer

preventing SMO from recruiting SUFU and other interacting proteins, with the effects of releasing GLI that translocates into the nucleus and activate target genes transcription (Fig.7) [56, 75, 76].

The role of SHH in MB was discovered by identifying inactivating mutations of the gene encoding PTCH1 on chromosome 22, resulting in the familial nevoid basal cell carcinoma (Gorlin's syndrome). Patients with Gorlin's syndrome are prone to develop MB [77, 78]. The role of SHH in MB tumorigenesis is supported by several observations. Aberrant SHH pathway activation by genetic mutations occurs in more than 15% of MBs. Mutations of PTCH1 occur in 10% of cases, whereas SMO activating mutations in 5% of cases [79, 80]. SUFU mutations have also been sporadically reported [81]. Deletion of chromosome 17p, the most commonly aberrant chromosome in MB, is associated with a reduced expression of the human orthologue of mouse REN (KCTD11), a negative regulator of tumor growth that inhibits SHH [82]. Moreover, mice in which the SHH pathway is aberrantly activated developed cerebellar tumors that resemble the histologic features and the genotype of human MB [68, 83].

Recent studies using unsupervised hierarchical cluster analysis of gene expression microarray data from two independent series of MBs showed that approximately 25% of the tumors were associated with activation of members and targets of the SHH pathway [39, 40]. SHH pathway activation is often observed in nodular/desmoplastic MBs [84].

Wingless pathway

WNT is a family of growth factor receptors acting through gene regulation and cell-cell control mechanisms. The WNT signaling is crucial for normal cerebellum development because it coordinates fate and proliferation of neural progenitor cells [70]. WNT regulates intra-cellular localization of the nuclear transcription factor β-catenin (Fig.7). Upon binding of WNT proteins to their receptor Frizzled (FRZ), a signaling cascade involving glycogen synthase kinase 3 (GSK3), adenomatous polyposis complex (APC) and AXIN1, takes place preventing proteosomal degradation of β-catenin [70, 85]. Consequently, β-catenin translocates into the nucleus and activates the DNA-binding HMG box lymphoid enhancer binding factor 1/T cell-specific factor (LEF1/TCF) transcription factors, inducing in turn the expression of downstream genes, such as *Cyclin D1* and *MYC* (Fig.7) [86].

The involvement of deregulated WNT pathway in MB emerged from genetic studies on the Turcot's syndrome, a tumor predisposition syndrome characterized by the development of

bowel tumors and increased frequency of primary CNS tumors, in particular MB and glioblastoma [87].

A significant subset of MBs is characterized by an aberrant activation of the WNT pathway [39, 40]. Approximately 10% of MB cases contain mutations in the gene encoding β-catenin (*CTNNB1*); aberrant stabilization of nuclear β-catenin protein occurs in 18–30% of cases [88]. Nuclear β-Catenin accumulation is associated with a better overall and event-free survival and it has been shown to be an independent marker of favorable clinical outcome [29, 88]. Mice with deletion of WNT1 do not show any cerebellar development, supporting the idea of a critical role for WNT signals in regulating cerebellum precursor cells [89, 90].

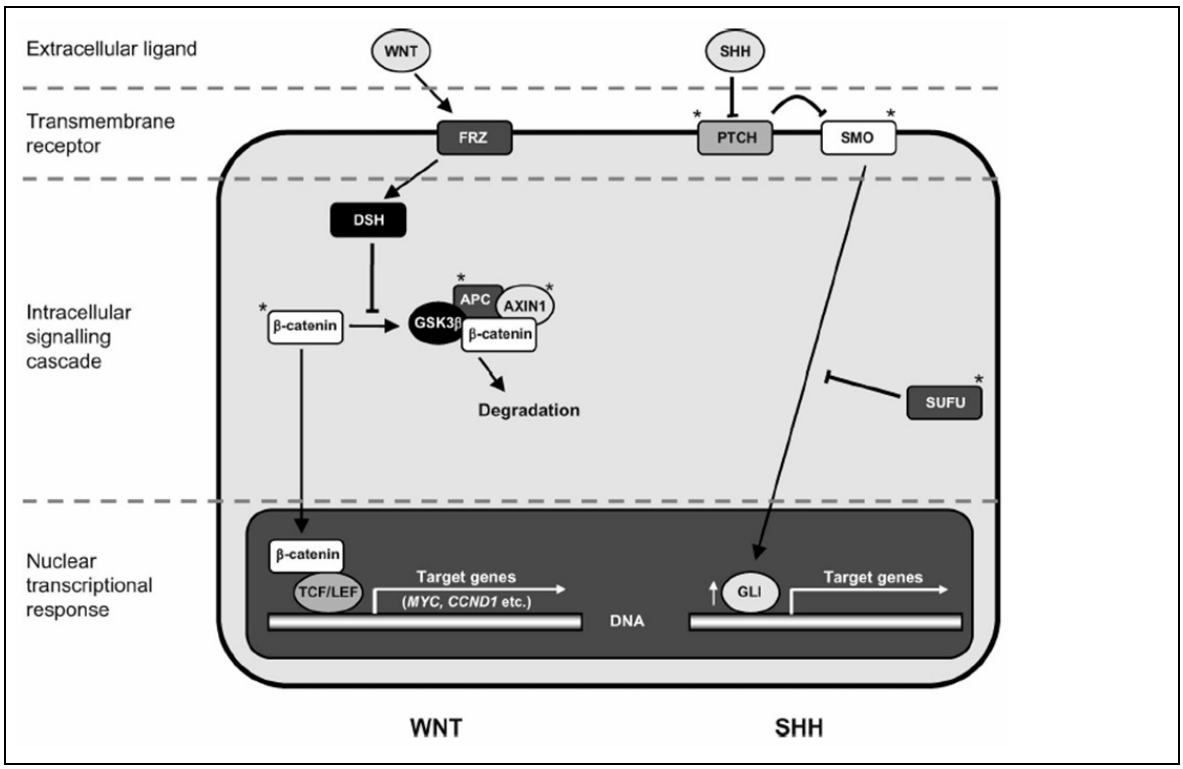

Figure 7. SHH and WNT pathways in medulloblastoma and mutations in specific proteins associated with pathway activation. Extracellular ligands WNT (wingless) and SHH (sonic hedgehog); transmembrane receptors FRZ (Frizzled) and PTCH (Patched); intracellular signaling cascade and nuclear transcriptional response. *Mutations in specific proteins associated with pathway activation, which have been reported in MB. **(Adapted from [10])**

Receptor tyrosine kinases

Receptor tyrosine kinases (RTK) are a large family of cell surface receptors with intrinsic protein tyrosine kinase activity [91]. RTK are activated by a variety of ligands and have pleiotropic effects on cell behavior, controlling cell-cycle, cell growth, proliferation, differentiation, cell migration, and apoptosis. Aberrations of members of the RTK family are

associated with MB development and have been considered for development of novel therapeutic strategies for MB (Fig.6) [92-94].

TrkC was the first RTK member to be significantly associated with prognosis in MB. TrkC mRNA expression is a powerful predictor of a favorable clinical outcome in MB patients [92, 95, 96]. Based on the intra-nodular localization observed in nodular/desmoplastic MB, TrkC signaling appears to be involved in apoptosis and neuronal differentiation of MB cells. MB patients expressing functional TrkC may be more prone to programmed cell death or to terminal neuronal differentiation [64].

Erythroblastic leukemia viral oncogene homolog 2 (neuro/glioblastoma derived oncogene homolog) (*ErbB2*, also known as *HER2/neu*) encodes a member of the epidermal growth factor (EGF) receptor family of RTKs. ErbB2 is expressed in more than 86% of MBs and several studies demonstrated that elevated expression of ErbB2 is associated with reduced MB patients' survival [97]. ErbB2 can up-regulate the expression of pro-metastatic genes, thus leading to a more aggressive tumor behavior (Fig.6) [93]. Various approaches targeting ErbB2 are currently under investigation (e.g. ErbB tyrosine kinase inhibitors and monoclonal antibodies, e.g. Trastuzumab) [93].

Mesenchymal epithelial transition factor (c-Met), also known as hepatocyte growth factor receptor (HGFR), is a proto-oncogenic RTK (Fig.6). c-Met mRNA expression levels significantly correlate with MB poor clinical outcome. c-Met and its ligand hepatocyte growth factor (HGF) play an important role in promoting MB malignancy. In particular, HGF induces tumor cell proliferation, anchorage-independent growth, and cell-cycle progression beyond the G1-S checkpoint. HGF also protected MB cells against apoptosis induced by chemotherapy. Moreover, MB xenograft models with HGF-over-expression exhibit increased tumor cells' invasion and morphologic changes that resembled human LCA MB [98].

Notch pathway

Notch signaling pathway is essential for proper development during embryogenesis. Various components of this pathway are expressed in the developing cerebellum and the signaling appears to be crucial in determining the cerebellar GNP cells' fate and proliferation. The cross-talk of the Notch cascade with the other main signaling pathways implicated in the cerebellum development orchestrates the entire network of signaling molecules involved [64, 99, 100].

Notch ligands (Jagged-1 and -2) bind to one of four notch receptors (Notch1–4) inducing the cleavage of Notch intracellular domain (NCI). The released NCI translocates into the nucleus, forms a complex with DNA binding proteins (CBF1), and activates the transcription of effector genes (Fig.6) [101].

Aberrant activation of the Notch pathway is associated with MB tumorigenesis. Notch1 was found amplified in 15% of MBs and Notch2 is expressed at high levels in a subset of tumors and it is associated with poor prognosis. Expression of truncated, constitutively active Notch2 in embryonal brain tumor cell lines induces an increase of cell proliferation, soft agar colony formation, and xenograft tumor growth [47].

MYC

MYC [*v-myc* myelocytomatosis viral oncogene homolog (avian)] is a family of genes *(c-MYC*, *MYCN*, *L-MYC)* which are key regulators of many biological processes, including cell growth and division (regulation of chromatin modification), cell-cycle progression (modulation of cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors and phosphatases), cell differentiation (modulation of growth arrest genes) and cell metabolism (glycolysis, amino acid biosynthesis and transport, synthesis of macromolecules and DNA metabolism) [102-105].

MYC is expressed during embryogenesis and in adult tissues with high proliferative capacity (such as skin, epidermis, and gut) [106] and it is almost undetectable in quiescent adult cells [107]. *MYCN* shows the highest mRNA expression in embryonal brain, kidney, and lung [108], whereas *c-MYC* levels are comparatively constant in these organs throughout development [109]. The knockout of either *MYC* gene resulted to be lethal *in vivo*. The mouse embryos die in the earliest developmental phases, confirming the essential role of these genes during mammalian embryogenesis [110, 111].

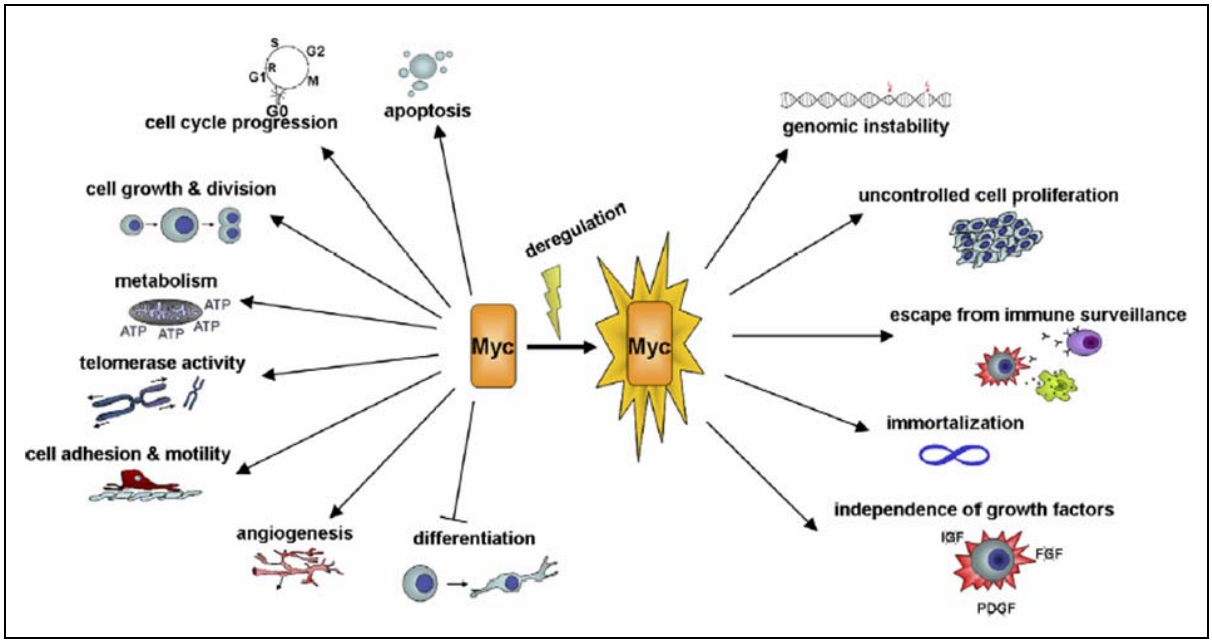

Figure 8. Cellular processes controlled by MYC during normal condition and during tumorigenesis. Myc is a key regulator of many biological activities including cell growth and division, cell-cycle progression, apoptosis; cell differentiation, cell metabolism, angiogenesis, cell adhesion and motility. Deregulation of Myc may result in apoptosis, genomic instability, uncontrolled cell proliferation, escape from immune surveillance, growth factor independence, and immortalization. **(Adapted from [105])**

MYC is a pleiotropic multifunctional transcription factor, responsible for activation and repression of a large number of target genes [112]. Analysis of the *in vivo* MYC-binding sites in the genome showed that MYC regulates about 10-15% of genomic loci in mammals [113, 114].

Through the transcriptional activation/repression of its target genes in a context-specific manner, MYC modulates diverse, sometimes opposing, groups of cellular processes. Up-regulated genes are mostly promoting cell growth, such as those involved in ribosome biogenesis, protein synthesis, and metabolism. Most frequently down-regulated genes are involved in cell-cycle arrest, cell adhesion, and cell-cell communication. The ability of MYC to stimulate cell growth and abrogate cell-cycle inhibitors is a crucial function for both normal development and tumorigenesis [115, 116].

During the life of a normal cell, the expression of *MYC* genes is strictly regulated. However, it can be abnormally activated by gene amplification, chromosomal translocation, DNA rearrangement, transcriptional up-regulation, and point mutation, all events leading to a constitutive over-expression of the oncogene [103, 117]. As a result of *MYC* de-regulated expression, tumorigenesis can be induced [118].

Deregulation of *MYC* contributes to several aspects of tumor cell biology: uncontrolled cell proliferation, inhibition of cell differentiation, induction of growth factor independency, neo-vasculogenesis, cell adhesion, promotion of metastasis, genomic instability, escape from immune surveillance, and immortalization [105, 111, 112, 119-121]. Elevated or deregulated expression of *MYC* family members has been detected in a wide range of human cancers, and they are frequently associated with aggressive and poorly differentiated tumors [103, 122].

MYC proteins consist of an N-terminal transactivation domain (TAD), responsible for assembly of the transcription machinery, a central region implicated in transcriptional repression, apoptosis, transformation, and DNA binding, and a C-terminal domain (CTD) that is critical for DNA binding and protein interactions [123, 124]. The CTD encompasses a basic region, required for binding to the consensus E-box elements, and a helix-loop-helix leucine zipper (HLHz) domain, necessary for dimerization with the partner protein MAX [124].

MYC-MAX heterodimers activate transcription of target genes [125] by binding to CACA/GTG sequences (a subset of the general E-box consensus sequence CANNTG, recognized by all HLHz proteins [126]). MYC-MAX heterodimerization is required for binding not only to the E-box sequence, but also to non-consensus binding sites. In fact, only

a minority of MYC-MAX binding sites *in vivo* contain the consensus sequence CACGTG. Other transcription factors (e.g. specificity protein-1 (SP1), nuclear factor Y (NF-Y), and transcription factor II-I (TFII-I)) are required for recruitment of MYC-MAX to non-consensus binding sites [127-129].

MAX can also form homodimers or heterodimers with members of the MAD family of proteins (MAD1, MXI1/MAD2, MAD3, MAD4 and MNT). MAD is a group of related HLHz proteins which compete with MYC for binding available MAX molecules, thus decreasing formation of MYC-MAX complexes [125, 130, 131].

MYC-MAX complexes are often predominant in proliferating cells, whereas complexes between MAX and MAD or MNT are predominant in resting or differentiated cells [112, 125].

MIZ1 is a zinc finger protein that modulates the biological function of MYC, binding to the HLH domain of MYC. Specific binding and inhibition of the transcriptional activator MIZ1 is one of the main mechanisms involved in MYC-mediated gene repression [132, 133].

A non-transcriptional function of MYC on chromatin was postulated based on the finding that about half of all MYC-bound sites are intergenic (>10 kb away from transcriptional start sites) [134]. Apparently the relative concentration of MYC and MAD proteins takes part in the determination of the active or inactive state of the chromatin for gene transcription. The transcriptional activation by MYC involves recruitment of co-activator molecules, including transformation/transcription domain-associated protein (TRRAP) and histone acetyl transferases (HAT) [135]. TRRAP is an adapter protein found in various multiprotein chromatin complexes with histone acetyltransferase activity, which gives a specific tag for epigenetic transcription activation. HATs-mediated acetylation of NH2-terminal lysine residues in the histones is associated with a more open (active) local chromatin structure, i.e. permissive conformation for enhanced transcription of specific target genes. On the other hand, MAD-MAX dimers recruit histone deacetylases (HDAC) [136] to the same E-boxes previously occupied by MYC-MAX dimers, thus leading to histone deacetylation and a more condensed (inactive) local chromatin structure. During tumorigenesis, aberrant high levels of MYC increase the expression of target genes that contribute to lock the chromatin in an abnormal active state. Data supporting this hypothesis showed that cells lacking the oncogene present more condensed chromatin structure, whereas MYC over-expression consistently leads to histone hyperacetylation [137].

MYC deregulation in medulloblastoma

Amplification of *c-MYC* in a MB cell line (D341) was discovered in 1988 by Friedman *et al*. [138]. Subsequently, several reports on amplification of both *c-MYC* and *MYCN* in MB followed [8, 21, 23, 37, 75, 139, 140]. *MYC* is expressed in up to 64% of MBs and amplified in 5 to 10% of MB patients, with this rate increasing to 17% in the high-risk sub-group of patients [33, 64]. Amplification of the *c-MYC* gene and high levels of the gene product have both been used as clinical indicators of poor prognosis [23, 95]. In contrast, low expression of *c-MYC*, alone or in combination with high *TrkC* mRNA levels, has been proved to be strong predictors of a favorable outcome in MB [23, 92].

The LCA phenotype is a histo-pathological prognostic factor for MB, indicative of a very aggressive clinical behavior and outcome [37, 141]. Different groups associated the amplification of *c-MYC* gene locus with MB LCA phenotype [21, 33, 142, 143]. Stearns *et al*. showed that increased c-MYC activity induces anaplasia and they established a causative link between *c-MYC* and anaplastic changes in MB [37]. MB cell lines engineered to stably overexpress the oncogene showed increased rates of growth, apoptosis, and ability to form colonies. *In vivo*, *c-MYC* over-expression in xenograft models led to development of significantly larger tumors with a cellular phenotype resembling the LC/A subtype of MB. The critical role of *c-MYC* in the development of LCA MBs was described as a multistep process, in which the oncogene may play a role in the initiation of some MBs, and its increased expression and activity is primarily observed as tumors progress and become more aggressive [37].

Although MYC does not represent a signaling pathway “per se”, it is a central mediator of almost all the pathways involved in cerebellar development and MB onset, including SHH, WNT, c-Met, Notch, and IGF-R (Fig.6) [7, 64].

MYC mRNA and protein levels increase rapidly upon mitogenic stimulation, driven by growth factors, mitogens, and other transcription factors like β-catenin [7, 64]. Also c-Met induces c-MYC. In particular, HGF induces cell-cycle progression, cell proliferation, apoptosis, and increase in cell size in a MYC-dependent manner, regulating MYC levels via transcriptional and post-transcriptional mechanisms [64, 98].

Moreover, *MYCN* modulates the effects of SHH activation on the proliferation of GNP cells, acting as the main conveyor of SHH-directed mitogenic activity, through the induction of Cyclin-D1 and Cyclin-D2 [59]. *MYCN* is up-regulated by SHH signaling through an increase of transcription and stabilization of MYCN protein [144], thus altering the regulation of the

cell-cycle [145]. Although the mechanism is not yet fully understood, it appears that SHH signaling increases activation of PI3K, which inhibits GSK-3β via phosphorylation, in turn having the effect of stabilizing MYCN. Similarly, when SHH signaling is decreased, activated GSK-3β leads to phosphorylation and degradation of MYCN [72].

The correlation between MYC and MB is further supported by the critical role of MYC during cerebellum development, in particular determining the GNP cells' fate [146]. *In vivo*, *MYC* plays a role in maintaining the GNP cell population by directing a rapid cell turn-over, activating cellular metabolism and inhibiting differentiation. In mice, double knock-out (DKO) of *c-Myc* and *N-Myc* strongly impairs brain growth, particularly that of the cerebellum. Granule neurons are almost absent from the *Myc* DKO cerebellum and show an aberrant cell fate, failing to migrate inward from the EGL to the IGL, and undergoing an abnormal differentiation within the EGL [146]. Moreover, conditional inactivation of *MYCN* in neural progenitors leads to a smaller and disorganized cerebellum [115, 147].

Bone morphogenetic proteins

In 1965, Marshall Urist discovered that decalcified bone matrix fragments have bone induction activity when transplanted into rats and rabbits [148]. More than 20 years later, the proteins capable of inducing bone and cartilage formation were isolated and named bone morphogenetic proteins (BMP) [149]. Nowadays, about twenty different BMP members have been identified and sub-grouped according to their amino acid sequence similarity and function: BMP-2/4 group, BMP-5/6/7/8 group (osteogenic protein 1 group), growth and differentiation factor (GDF)-5/6/7 group and BMP-9/10 group (Fig.9) [150]. Additional to induction of bone and cartilage tissues, these proteins can regulate tooth, kidney, skin, hair, muscles, haematopoietic, and neuronal development [151, 152].

BMPs are cytokines belonging to the TGF-β family, which are able to regulate various cellular processes, including migration, proliferation, apoptosis, differentiation, iron metabolism, and vascular homeostasis [153, 154].

BMPs are generated as large precursor proteins composed of an N-terminal signal peptide, which directs the protein to the secretory pathway, a pro-domain that ensures proper folding, and a C-terminal mature peptide. Specific serine endo-proteases cleave the signal peptides. The generated mature ligand is secreted. Each monomer is stabilized by three intramolecular disulfide bonds, formed between six highly conserved cysteines (cysteine knot motif). The active forms of BMPs are homo- and hetero-dimers, covalently linked via a disulfide bond, requiring a seventh conserved cysteine within each monomer [153, 155].

BMPs act both in paracrine and autocrine manners to promote the recruitment of cell surface type I and type II serine/threonine kinase BMP receptors [154, 156]. Type I receptors include BMPR1A/Alk-3 (BMP receptor type 1A, also named Activin like kinase-3), BMPR1B/Alk-6 (BMP receptor type 1B, also named Activin like kinase-6), Alk-2/ActR1A (Activin like kinase-2, also named Activin receptor type 1A) and Alk-1. BMPR1A and Alk-2 are widely expressed in various types of cells. The expression of BMPR1B shows a more restricted expression profile and that one of Alk-1 appears to be limited to endothelial cells [157]. Type I receptors form heteromeric complexes with three type II receptors which are widely expressed in mammals' tissues: BMP receptor type II (BMPRII), Activin receptors type IIA and type IIB (ActRII or Acvr2A and ActRIIB or Acvr2B) [155, 156]. Type I and type II receptors present structural similarities, including a short extracellular domain, a single membrane-spanning domain and an intracellular domain containing a serine-threonine kinase.

Upon ligand binding, type II receptors trans-phosphorylate the glycine/serine-rich region (GS-box) of the associated type I receptor which, in turn, transduces the signal activating downstream effector proteins [157, 158].

Unlike TGF-β, which displays high affinity for type II receptors and does not stably interact with type I receptors, BMPs bind independently and with different affinities to both type I and type II receptors [159]. In particular, BMP2 and BMP4 show high affinity for the type I receptors BMPR1A and BMPR1B [160, 161], whereas BMP7 preferentially binds to the type II receptors ActRIIA and ActRIIB [162]. These differences in specificity determine formation of distinct oligomers of receptors, thus leading to selective activation of specific signaling cascades [163-165].

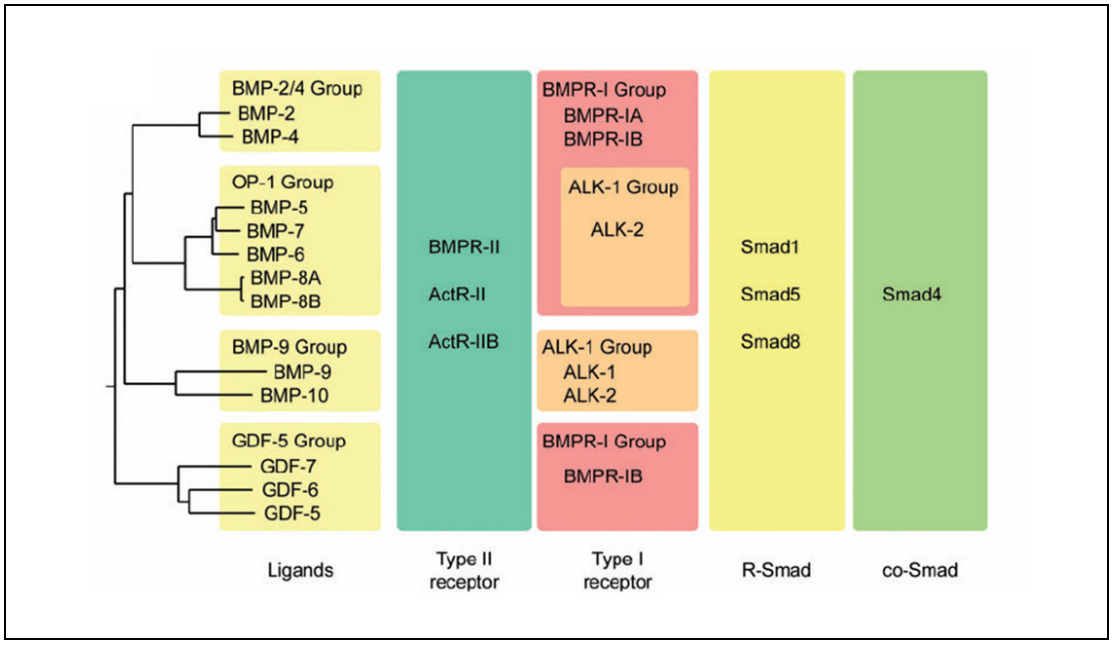

Figure 9. Relationship between BMPs, receptors and SMAD proteins in signal transduction. Relationships between subgroups of BMP ligands, type II and type I receptors, and Smad proteins in signal transduction. BMP-2/4 subgroup binds to BMPR-IA and BMPR-IB, whereas OP-1 subgroup binds strongly to ALK-2, and weakly to BMPR-IB. BMP-9/10 bind to ALK-1 and ALK-2, and GDF subgroup preferentially binds to BMPR-IB. R-SMADs and Co-SMADs are shared by all BMP ligands. **(Adapted from [156])**

Signal transduction: SMAD-dependent and SMAD-independent pathways and modulators.

SMADs are the major signal transducers for BMP receptors. They owe their name to the homology with Sma proteins from nematode *Caenorhabditis elegans* and Mad (mothers against decapentaplegic) proteins from the fly *Drosophila melanogaster*. SMADs are subdivided into three subtypes: receptor-activated SMADs (R-SMADs), common-mediator SMAD (Co-SMAD), and inhibitory SMADs (I-SMADs).

The R-SMAD (SMAD1, SMAD5, and SMAD8) activation is mediated by engagement of activated type I BMP receptors, which phosphorylate a conserved SSXS motif on the CTD of specific R-SMAD. BMPR1A/Alk-3 and BMPR1B/Alk-6 can activate all three SMADs (1/5/8), whereas Alk-2 can only phosphorylate SMAD1 and 5 [154].

SMAD4 is the Co-SMAD shared by BMP and TGF-β signaling pathways. In the cytoplasm, SMAD4 assembles with activated R-SMADs to form a heteromeric complex, which translocates into the nucleus to regulate gene transcription (Fig.10).

I-SMAD (SMAD6 and SMAD7) inhibit the phosphorylation of R-SMADs by competing with R-SMADs for the binding to phosphorylated type I receptors [151, 166].

R-SMADs contain two conserved domains, linked by a variable proline-rich region. The N-terminal MH1 domain is responsible for DNA binding and for other protein-protein interactions; the C-terminal MH2 domain contains the SXSS-motif mediating R-SMAD specificity, receptor recognition, nuclear translocation, and oligomerization [167]. Unphosphorylated, inactive R- SMADs adopt an autoinhibitory structure in which their MH1 and MH2 domains interact. Upon phosphorylation at the C-terminus, R-SMADs are activated and relieved from this structure. Subsequently, the signal peptide for nuclear translocation is exposed, the affinity for SMAD4 increases, and the complex translocates into the nucleus [166].

When cells are in a non-stimulated state, SMAD4 is uniformly distributed throughout the cell compartments, R-SMADs are principally located in the cytoplasm and the I-SMADs are mainly in the nucleus. Upon ligand binding, R-SMADs and SMAD4 accumulate in the nucleus, although they are constantly shuttled between the nucleus and the cytoplasm [168].

SMADs bind to DNA weakly. The required high affinity and specificity for the transcriptional activation are obtained through the involvement of additional DNA binding factors, co-repressors and activators [169].

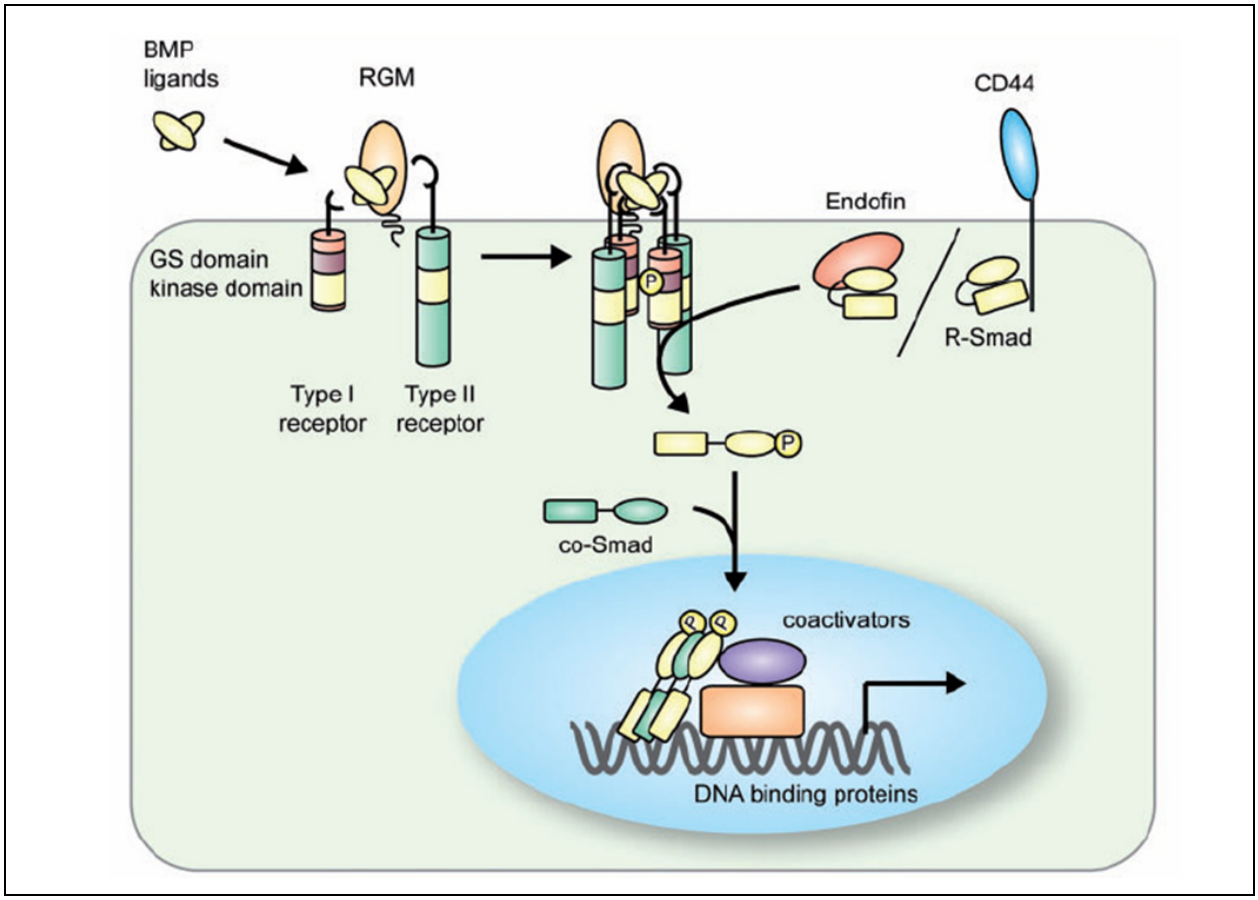

Figure 10. BMP signaling, from the plasma membrane to the nucleus. BMP ligands bind to heterotetrameric complex of type II and type I receptors, and RGM proteins serve as co-receptors for BMPs. R-SMADs exist in the cytoplasm through interaction with membrane anchoring proteins, e.g. CD44 and endofin. Upon phosphorylation by type I receptors, R-SMADs form complexes with co-SMAD (SMAD4), translocate into the nucleus and regulate transcription of target genes through interaction with transcription factors (DNA-binding proteins) and transcriptional co-activators. **(Adapted from [156])**

Although the SMAD pathway represents the main downstream signaling cascade, BMPs can also activate distinct SMAD-independent pathways (i.e. mitogen-activated protein kinase (MAPK) pathway) [154, 170].

The physiological responses to each BMP are tissue- and cell-specific. In fact, the microenvironment plays a key role in modulating the temporal and spatial expression of each BMP and its receptors. [155]. The presence or absence of a variety of antagonists [171, 172] and modulators [173, 174], and the concomitant cross-talk of the BMPs with other pathways (e.g. WNT, PI3K, and Notch) contribute to the regulation of the whole signaling network [154, 175].

The concentration of each BMP is a key factor for the biological responses. Several extracellular antagonists bind to specific BMPs regulating the bioavailability of each molecule for the receptors [176-178]. Like the BMP ligands, the antagonists are homodimeric proteins containing a cystine knot motif, which stabilizes their structure. Antagonists are

subgrouped according to their cystine knot motif: the Chordin/Noggin family, Twisted Gastrulation and the DAN/Cerberus family [179]. Other known BMP-antagonists are named Follistatin, Gremlin, Sclerostatin, Chordin-like, etc. Most of the antagonists appear to be synthesized together with BMP ligands and their expression is frequently controlled by the BMP itself, as a self-regulated feed-back loop [174].

The signal transduction is also regulated by many co-receptors. Co-receptors that potentiate BMP signaling include the repulsive guidance molecules (RGMa and RGMc), Dragon (RGMb), the RTK c-Kit, and the TGF-β/BMP type III receptors Endoglin and Betaglycan. BMPs are negatively regulated by the decoy-receptor BAMBI (BMP and activin membrane-bound protein) and the RTK Ror2 and TrkC (Fig. 10) [155].

BMPs: roles in tumorigenesis

Alterations of the BMP pathway have been described in several tumor systems including breast, prostate, ovarian, lung, and colon cancers, as well as in MB, glioblastoma, and osteosarcoma [180-187]. BMPs take part in the modulation of potential carcinogenic processes such as cell proliferation, apoptosis, differentiation, metastasis, and angiogenesis. Similarly to other TGF-β members, BMPs show a dual role in cancer development, showing both pro- and anti-tumorigenic effects [188, 189]. For instance, BMP signaling has been shown to inhibit tumorigenesis of several tumor types, including brain tumors, basal cell carcinomas of the skin and gastric cancer [185, 190, 191]. On the other hand, there are evidences showing that BMPs can promote proliferation, motility and invasiveness of various cancer models, including colon, melanoma and prostate cancer [192-195]. The discrepant data originates most likely from the complexity of the BMP signaling network, strongly dependent on the microenvironment. The physiological responses to each BMP are different at the molecular level, depending on the type of cell and/or tissue and can be widely altered by the aberrant tumor microenvironment [189, 196].

Prostate cancer studies have shown that expression of BMPs increases with the tumor progression. Furthermore, it is common, in advanced phases of prostate cancer, to find high expression of BMPs associated with bone metastasis [153]. In particular, BMP6 is detected exclusively in malignant epithelial prostate cells in patient samples with metastases, and in isolated skeletal metastases from prostate carcinomas [197].

BMPs have also been shown to have an impact on the behavior of breast cancer and the expression levels of components of the BMP signaling correlate with the degree of breast

cancer malignancy [198]. The analysis of the expression of BMPs in breast carcinomas demonstrated a differential expression pattern and their potential prognostic value [182, 196]. In particular, the BMP2 transcript level was found lower and the BMP7 level higher in breast tumors compared to normal tissues. The BMP2 transcript was also significantly lower in tumors from patients with moderate and poor prognosis than in those from patients with a good prognosis [153]. Moreover, several studies demonstrate the involvement of different BMPs and their receptors in highly metastatic breast cancer cells. BMP7 appears to be associated with accelerated bone metastasis in breast cancer [199].

Moreover, BMP7 is able to trigger proliferative defects in p53-deficent breast cancer cells when knocked-down [200], and to promote proliferation of some breast cancer cell lines [199]. However, other studies demonstrated a proliferation inhibitory effect of BMP7 showing that the expression is inversely correlated to tumorigenicity and invasiveness *in vitro* [187].

BMPs in cerebellar development and medulloblastoma onset

During embryonic development, organogenesis and tissue regeneration, BMPs take part to the regulation of the stem cell population, in particular maintaining embryonic stem cell self-renewal. Alterations of components of the BMP signaling may lead to uncontrolled proliferative activity of normal stem cells, breaking the fine balance between quiescence and proliferation and leading to accumulation of cells without a fully mature phenotype due to failure in the signal that drives differentiation [153].

During neural development in different regions of the CNS, BMPs control cell fate, proliferation and differentiation of several embryonic cell types to give rise to diverse components of the nervous system [201]. The knowledge about the roles of BMPs during development comes from observations of gene defects in different organisms and from gene knockout experiments in mice. Distinct phenotypes and malformations in different organs are associated with alteration of BMP signaling. Compound mutant mice for BMP2 and 4 show multiple defects in various organ systems, including skeleton, eye, and heart [202]. Homozygous null mutations of BMP7 result in skeletal defects as well as severe defects in kidney and eye development [203].

As mentioned in the section about normal cerebellum development, BMPs participate by regulating proliferation, cell-cycle exit, migration, and differentiation of GNP cells [204]. GNPs originate within the rhombic lip and BMP7, BMP6 and Gdf7 are expressed by roof

plate cells of the dorsal midline adjacent to the rhombic lip [54]. In particular, in the mouse embryo BMP7 is expressed within roof plate cells, whereas BMP6 and Gdf7 are expressed at highest levels in cells of the dorsal neuroepithelium adjacent to the roof plate (Fig.11) [54]. This region and the secreted signals are implicated in proliferation and migration of GNPs [205]. Furthermore, BMPs showed inductive activities on GNP cells, stimulating the generation of granule neurons cells *in vitro* and acting as survival factors for postnatal granule cells [7, 206, 207].

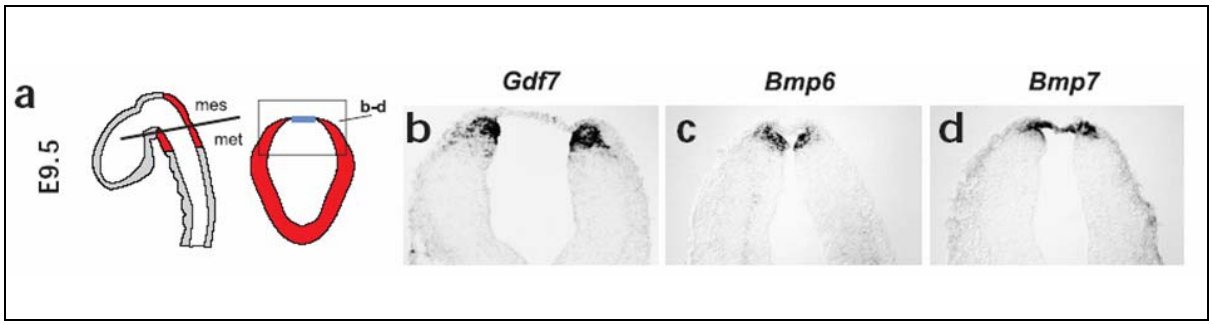

Figure 11. Expression of BMPs in the mesencephalon/metencephalon (mes/met) region roof plate. (a) Schematic diagram of an E9.5 mouse embryo, showing a sagittal section on the left and a transverse section on the right. Roof plate is represented in blue (b–d). Transverse sections through the mes/met region of E9.5 mouse embryos (boxed in a) showing localization of BMPs by in situ hybridization. Gdf7 (b), BMP6 (c), and BMP7 (d) are expressed along the dorsal edges of the neural tube. **(Adapted from [54])**

Moreover, the choroid plexus is a non-neuronal lobulated structure, composed of blood vessels enveloped by a monolayer of epithelial cells, which is located throughout the fourth ventricle, bordering the rhombic lip [208, 209]. Besides its secretory and barrier functions, the choroid plexus plays a role in early GNP specification maintaining these cells in a proliferative state and antagonizing differentiation before their migration towards the external and internal granule layer [50, 210]. BMP7 is secreted by cells of the choroid plexus and takes part to the inhibition of GNP cells differentiation in the rhombic lip. The mechanism of action appears to be related with the ability of BMP7 to increase the expression of MATH1, thus maintaining GNP cells in a MATH1 expressing, proliferative state [210]. Differentiation of GNPs into the IGL requires decreased levels of BMP7, achieved through MATH1-mediated induction of the BMP7 antagonist Nbl1 (Fig.12) [50].

Thus, BMP7 contributes to determining a correct temporal regulation and spatial organization of cerebellar progenitor cells during early development [40]. Along with this role, deregulated BMP7 expression in the cerebellum is very likely associated with abnormal cell development and MB tumorigenesis.

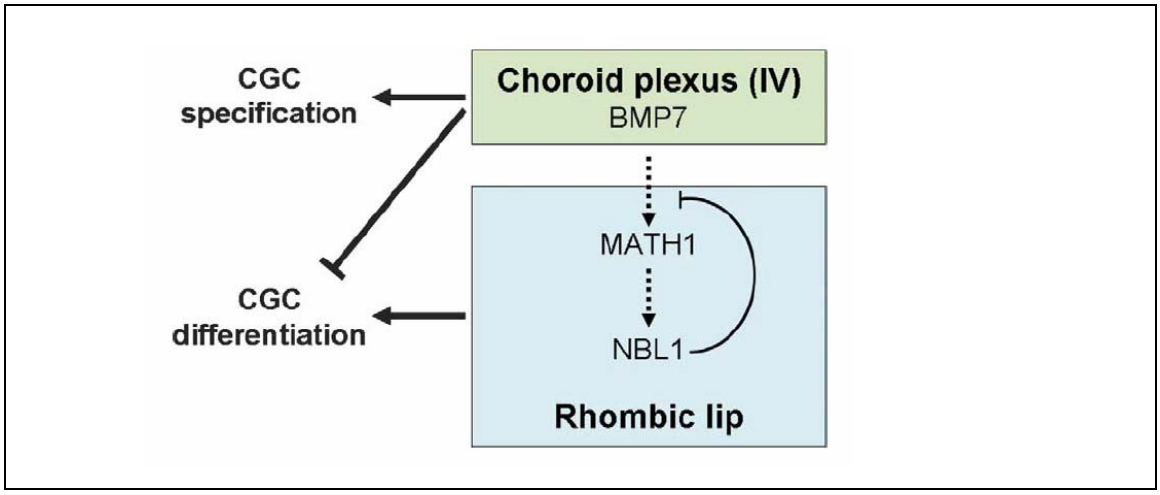

Figure 12. Schematic representation of the regulation of GNP cells development in the rhombic lip. BMP7 specifies the fate of rhombic lip progenitor cells as future granule neurons, inhibits their differentiation, and activates the expression of MATH1. Later, MATH1-mediated expression of Nbl1 antagonizes the effect of BMP7 and promotes differentiation. **(Adapted from [210]).**

Within the IGL, a distinct subgroup of BMPs (BMP2 and BMP4) take part to the cerebellar development, counteracting SHH signals and allowing differentiation of GNP cells (Fig.5) [62]. These BMPs are capable of inducing differentiation in a gradient-based manner. BMP2 expression increases in the IGL as it develops, while BMP4 is expressed at a low level and its expression and protein level peak during GNP differentiation and migration toward the IGL [211].

Besides their roles in GNP cells differentiation, several reports suggested that BMPs have an anti-apoptotic effect in many cell types [212, 213]. However, the anti-apoptotic effects of BMPs in the developing cerebellum and the mechanisms involved remain largely unknown.

Investigations about treatment of MB cells with BMP2 and BMP4 showed either attenuation of intrinsic apoptotic mechanisms and increases in cell count [214] or inhibition of proliferation [215]. Murine MB cells overexpressing BMP4 within a nude mouse model showed decreases in cellular proliferation. BMP2 and BMP4 also inhibited the rate of gross MB tumor growth [215].

Although the exact role of BMP7 in MB is not yet well characterized, the available information [50], along with our experimental results suggest that the contribution of BMP7 to MYC-dependent pro-survival mechanisms is important in MB cells.

Subject of Investigation

Identification of *MYC* target genes in childhood medulloblastoma and validation of Bone morphogenetic protein-7 as a direct *MYC* target with pro-survival functions.

A better knowledge of the *MYC* effector genes involved in MB neoplastic transformation is of great relevance and can suggest novel and suitable therapeutic strategies. To identify MYC transcriptional targets and their roles during MB onset and progression we compared the cDNA microarray profiles of DAOY-derived cells upon genetic manipulation aiming at either overexpressing or downregulating *MYC*. We identified 209 genes with potential relevance to MYC-dependent cellular responses in MB. Among the MYC-responsive genes, we found members of the BMP signaling pathway. In particular, we identified the gene encoding for the cytokine BMP7 as a direct target of MYC. We confirmed the binding of MYC to the promoter of BMP7 by ChIP-on-chip analysis in different MB cell lines. Moreover, BMP7 is the only member presenting a positive correlation with *MYC* in human MB patient samples. Next, we evaluated the functional relevance of the BMP pathway in MB by employing a small-molecule inhibitor of SMAD1/5/8 activation/phosphorylation (DM). DM induces apoptosis and decreases cell viability of MB cells. DAOY cells expressing a single *MYC* copy are significantly less sensitive to DM than cells overexpressing the oncogene, indicating that the compound can affect a MYC-driven pro-survival response. Similarly to DM, BMP7 silencing by siRNA increased apoptosis and reduced cell viability of MB cells. Altogether, our findings indicate that high MYC levels drive BMP7 expression in MB to induce pro-survival and pro-proliferative cellular pathways. This observation suggests the potential relevance of this growth factor as a MYC effector protein during MYC-driven neoplastic transformation of MB precursor cells. This new information also suggests a rationale for targeting BMP pathways as a therapeutic intervention strategy for MB patients with *MYC* amplification/overexpression.

In this manuscript, I developed the idea behind the investigation. I performed all the experiments shown in figure 1, 3e, 4, 5, 6, table 1 and in supplementary figures and tables. Figures 2 and 3 (a, b, c, d) were obtained through the collaboration with the group of Frank Westermann (Dept. Tumor Genetics, German Cancer Research Center (DKFZ) Heidelberg, Germany) and

the group of Hiroko Ohgaki (Section of Molecular Pathology, International Agency for Research on Cancer (IARC), World Health Organization (WHO), Lyon, France) respectively. I wrote the text under the supervision of Michael Grotzer.

The quassinoid derivative NBT-272 targets both the mTOR and Erk signaling pathways in embryonal tumors

In the context of *MYC*-overexpressing embryonal tumors (ET), we wanted to investigate the biological responses of a group of ET-derived cell lines to the quassinoid analogue NBT-272, a small molecule described as inhibitor of MYC. NBT-272 displayed a strong anti-proliferative activity and the ability to prevent colony formation in vitro, resulting from the combination of diverse biological effects, ranging from G1/S arrest of the cell-cycle to apoptosis and autophagy. We investigated the mechanisms of action of NBT-272 and found that it is able to prevent full activation of both the eukaryotic initiation factor 4E and its binding protein 4EBP-1 that regulate cap-dependent protein translation. Moreover, NBT-272 significantly interfered with full activation of two key pro-proliferative signaling pathways, i.e. the Akt/mammalian target of rapamycin (mTOR) and the Mek/extracellular signal-regulated kinase (Erk) pathways. It is likely that both these signaling pathways participate in orchestrating the cellular responses to NBT-272 and that the depleting effect of NBT-272 on MYC protein expression occurred via indirect mechanisms, rather than selective inhibition. Further, we checked whether the effects could be reproduced *in vivo* and found that NBT-272 treatment arrests tumor growth in a xenograft model of human NB. Moreover, expression of MYC and activation of Erk were reduced significantly in the tumors treated with NBT-272. In summary, NBT-272 induces a relatively complex pattern of cellular responses. All these events could be related functionally to interference with key cell survival pathways playing a role in the pathogenesis of several ETs. This study justifies further efforts to define more clearly the potential benefits of using NBT-272 in novel therapeutic strategies for pediatric tumors.

In this manuscript, I contributed to the development of the idea behind the investigation and to the writing of the text. I performed the experiments involving the dose-dependent effect of NBT-272 on cell viability (Fig. 1B) and the anchorage-independent growth in soft agar (Fig.1C) of ET-derived cell lines; I contributed to the creation of table 1; I performed the apoptosis analysis shown in figure 3A; I performed the analysis of mRNA levels (qRT-PCR) (Fig. 4A - bottom panel and supplementary Fig. 1SB) and the RT2 Profiler PCR Array (Fig.4D and supplementary Fig.4S); I performed the experiments involving the evaluation of the cell viability in NB-derived cell lines treated with Mycro3 and 10058-F4 (Fig. 4C); I performed the experiment involving the siRNA-mediated silencing of MYC shown in supplementary figure 6S.

Disabling c-MYC in Childhood MB and Atypical Teratoid/Rhabdoid Tumor Cells by the Potent G-Quadruplex Interactive Agent S2T1-6OTD

S2T1-6OTD is a novel telomestatin derivative synthesized to target G-quadruplex–forming DNA sequences. S2T1-6OTD strongly inhibits c-MYC through its high-affinity physical interaction with the G-quadruplex structure in the c-MYC promoter. We investigated the efficiency of S2T1-6OTD to stabilize G-quadruplexes in guanine-rich DNA and examined the strength of its selectivity for the c-MYC over the telomeric sequences. We found that, through its ability to recognize and stabilize the quadruplex structure in the c-MYC promoter sequence, S2T1-6OTD is able to reduce the mRNA and protein expressions and decreased the activities of c-MYC and its target gene *hTERT*. Further, we investigated the effects of S2T1-6OTD on a representative panel of human MB and atypical teratoid/rhabdoid (AT/RT) childhood brain cancer cell lines. Treatment with S2T1-6OTD resulted in a dose and time-dependent antiproliferative effect in all cell lines tested. Moreover, S2T1-6OTD treatment induced cell-cycle arrest and decreased the protein expression of the cell-cycle activator cyclin-dependent kinase 2. Long-term treatment with nontoxic concentrations resulted in a time-dependent telomere shortening, preceded by a decrease in telomerase activity, and followed by cell growth arrest, cell senescence and induction of apoptosis. Altogether our findings suggest that S2T1-6OTD may well represent a novel therapeutic strategy for childhood brain tumors.

My contribution to this manuscript involved the analysis of mRNA levels (RT-PCR measurements) of MB cells (Fig.1A – upper panel), the analysis of cell viability (Fig.5A) and the measurement of apoptotic cell death (Fig.6C).

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ORIGINAL ARTICLE

Bone morphogenetic protein-7 is a MYC target with prosurvival functions in childhood medulloblastoma

G Fiaschetti[1], D Castelletti[1], S Zoller[2], A Schramm[3], C Schroeder[4], M Nagaishi[5], D Stearns[6], M Mittelbronn[7], A Eggert[3], F Westermann[4], H Ohgaki[5], T Shalaby[1], M Pruschy[8], A Arcaro[9] and MA Grotzer[1]

[1]*Department of Oncology, University Children's Hospital, Zurich, Switzerland;* [2]*Functional Genomics Center Zurich, UZH/ETH, Zurich, Switzerland;* [3]*Division of Hematology/Oncology, University Children's Hospital Essen, Essen, Germany;* [4]*Department Tumor Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany;* [5]*Section of Molecular Pathology, International Agency for Research on Cancer, World Health Organization, Lyon, France;* [6]*Department of Pathology, Johns Hopkins University, Baltimore, MD, USA;* [7]*Institute of Neurology (Edinger Institute) Goethe-University Frankfurt, Frankfurt/Main, Germany;* [8]*Department Radiation Oncology, University Hospital, Zurich, Switzerland and* [9]*Division of Pediatric Hematology/Oncology, Department of Clinical Research, University of Bern, Bern, Switzerland*

Medulloblastoma (MB) is the most common malignant brain tumor in children. It is known that overexpression and/or amplification of the MYC oncogene is associated with poor clinical outcome, but the molecular mechanisms and the MYC downstream effectors in MB remain still elusive. Besides contributing to elucidate how progression of MB takes place, most importantly, the identification of novel MYC-target genes will suggest novel candidates for targeted therapy in MB. A group of 209 MYC-responsive genes was obtained from a complementary DNA microarray analysis of a MB-derived cell line, following MYC overexpression and silencing. Among the MYC-responsive genes, we identified the members of the bone morphogenetic protein (BMP) signaling pathway, which have a crucial role during the development of the cerebellum. In particular, the gene *BMP7* was identified as a direct target of MYC. A positive correlation between *MYC* and *BMP7* expression was documented by analyzing two distinct sets of primary MB samples. Functional studies *in vitro* using a small-molecule inhibitor of the BMP/SMAD signaling pathway reproduced the effect of the small interfering RNA-mediated silencing of *BMP7*. Both approaches led to a block of proliferation in a panel of MB cells and to inhibition of SMAD phosphorylation. Altogether, our findings indicate that high MYC levels drive *BMP7* overexpression, promoting cell survival in MB cells. This observation suggests the potential relevance of targeting the BMP/SMAD pathway as a novel therapeutic approach for the treatment of childhood MB.
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Keywords: BMP7; MYC; medulloblastoma; brain tumor; pediatric cancer

Correspondence: Professor Dr MA Grotzer, Department of Oncology, University Children's Hospital, Steinwiesstrasse 75, Zurich CH-8032, Switzerland.
E-mail: Michael.Grotzer@kispi.uzh.ch
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Introduction

Medulloblastoma (MB) represents >20% of all pediatric tumors of the central nervous system (Gurney and Kadan-Lottick, 2001), and is characterized by aggressive clinical behavior and high risk of leptomeningeal dissemination (Engelhard and Corsten, 2005). Multimodal therapy (surgery, radiotherapy and chemotherapy) has improved the overall survival rate. However, about 30% of patients are still incurable, and survivors often suffer from severe tumor- and therapy-related cognitive and neurological dysfunctions (Mulhern *et al*., 2005). MB can be subdivided into different histological variants (Eberhart *et al*., 2002; Lamont *et al*., 2004); the large-cell/anaplastic MB (20–25% of patients) represents the most aggressive subtype, and is associated with a high degree of genomic instability and deregulated expression of the oncogene *MYC* (Stearns *et al*., 2006; Gilbertson and Ellison, 2008).

MYC represents a family of pleiotropic transcription factors that activate/repress a large group of target genes (Grandori *et al*., 2000; Adhikary and Eilers, 2005) that are, in turn, the effectors of MYC-dependent biological responses, ranging from cell growth, proliferation and migration to programmed cell death and differentiation. MYC is primarily expressed during development and is mainly repressed in adult tissues, in which aberrant expression (upregulation) can drive neoplastic transformation (Littlewood and Evan, 1990). In MB, MYC upregulation can originate from genomic amplification (4–10% of MB patients; Neben *et al*., 2004) or mRNA overexpression (30–65% of patients; Herms *et al*., 2000; Aldosari *et al*., 2002), and it can occur as a downstream effect of deregulated signaling pathways (Guessous *et al*., 2008) that are involved in the development of granule neuron precursor cells (GNPs) of the cerebellum (Gilbertson and Ellison, 2008).

Different approaches have been evaluated to target MYC in various malignancies, including MB (Vita and Henriksson, 2006; Grotzer *et al*., 2009). However, direct pharmacological targeting of transcription factors has

proven difficult, thus warranting the effort of investigating in more detail genes that are downstream of MYC as novel therapeutic targets. Furthermore, a selective targeting of downstream effectors would aim at preserving the anti-proliferation (for example pro-apoptotic) functions of MYC.

In this study, we combined gene expression data from MB-derived cell lines, which had been engineered to either overexpress or silence *MYC*. Among the MYC-responsive genes, we searched for candidate drivers of MYC-dependent tumorigenesis in MB. The results suggested the possible relevance of bone morphogenetic proteins (BMPs), which are cytokines of the family of the transforming growth factor-β (Blanco Calvo *et al*., 2009). BMPs can be activated, both in a paracrine and autocrine way, to promote recruitment of cell surface serine/threonine kinase receptors, thus triggering activation of SMAD transcription factors (Miyazono *et al*., 2005, 2010).

Many studies report on alterations of the BMP pathway in several tumor systems, including breast, prostate, lung, and colon cancers, as well as glioblastoma, osteosarcoma and MB (Arihiro and Inai, 2001; Langenfeld *et al*., 2003; Miyazaki *et al*., 2004; Raida *et al*., 2005; Piccirillo and Vescovi, 2006; Ye *et al*., 2007; Buijs *et al*., 2007a; Dai *et al*., 2008). Similar to other transforming growth factor-β members (Derynck *et al*., 2001), BMPs may have a dual role in cancer, both promoting and repressing tumorigenesis in several models (Buijs *et al*., 2007a, b; Ye *et al*., 2009; Thawani *et al*., 2010). BMP7, for instance, was shown to promote migration and invasion of prostate cancer cells (Feeley *et al*., 2005), but also to inhibit prostate tumor cell growth *in vivo* (Buijs *et al*., 2007b). Miyazaki *et al*. (2004) showed a dose-dependent effect of BMP7, which inhibited proliferation of androgen-insensitive prostate cells at high concentrations, whereas low doses had a pro-proliferative effect. In breast cancer, the expression level of *BMP7* was found inversely correlated to tumorigenicity and invasiveness *in vitro* (Buijs *et al*., 2007a), but it was also found to cause abnormal proliferation in p53-deficient breast cancer cells when knocked down (Yan and Chen, 2007). Although apparently contradictory, these data reflect the complexity of the BMP/SMAD signaling pathway (Ye *et al*., 2009), leading to diverse heterogeneous cellular responses, which can be tissue- and cell type-specific and result from the cross-talk with the microenvironment. This latter heavily influences the temporal and spatial expression of BMPs (Sieber *et al*., 2009), implying also that co-expression of antagonists (Walsh *et al*., 2010; Yanagita, 2005) or modulators (Balemans and Van Hul, 2002; Yanagita, 2009), as well as the activation status of other pathways (Miyazono *et al*., 2005; Guo and Wang, 2009), all contribute significantly to the diverse responses observed.

Finally, because of the relevance of BMPs during cerebellum development (by regulating proliferation, cell cycle exit, migration and differentiation of GNPs; Gilbertson and Ellison, 2008), it is not surprising that BMPs are likely to have a role in tumorigenesis and at least in a subset of MB tumors (Thompson *et al*., 2006; Grimmer and Weiss, 2008; Behesti and Marino, 2009; Sutter *et al*., 2010). In this respect, our experimental results are consistent with this biological hypothesis and provide an important contribution by linking, for the first time, a BMP family member to the oncogene MYC, in the context of MYC-dependent prosurvival mechanisms in MB.

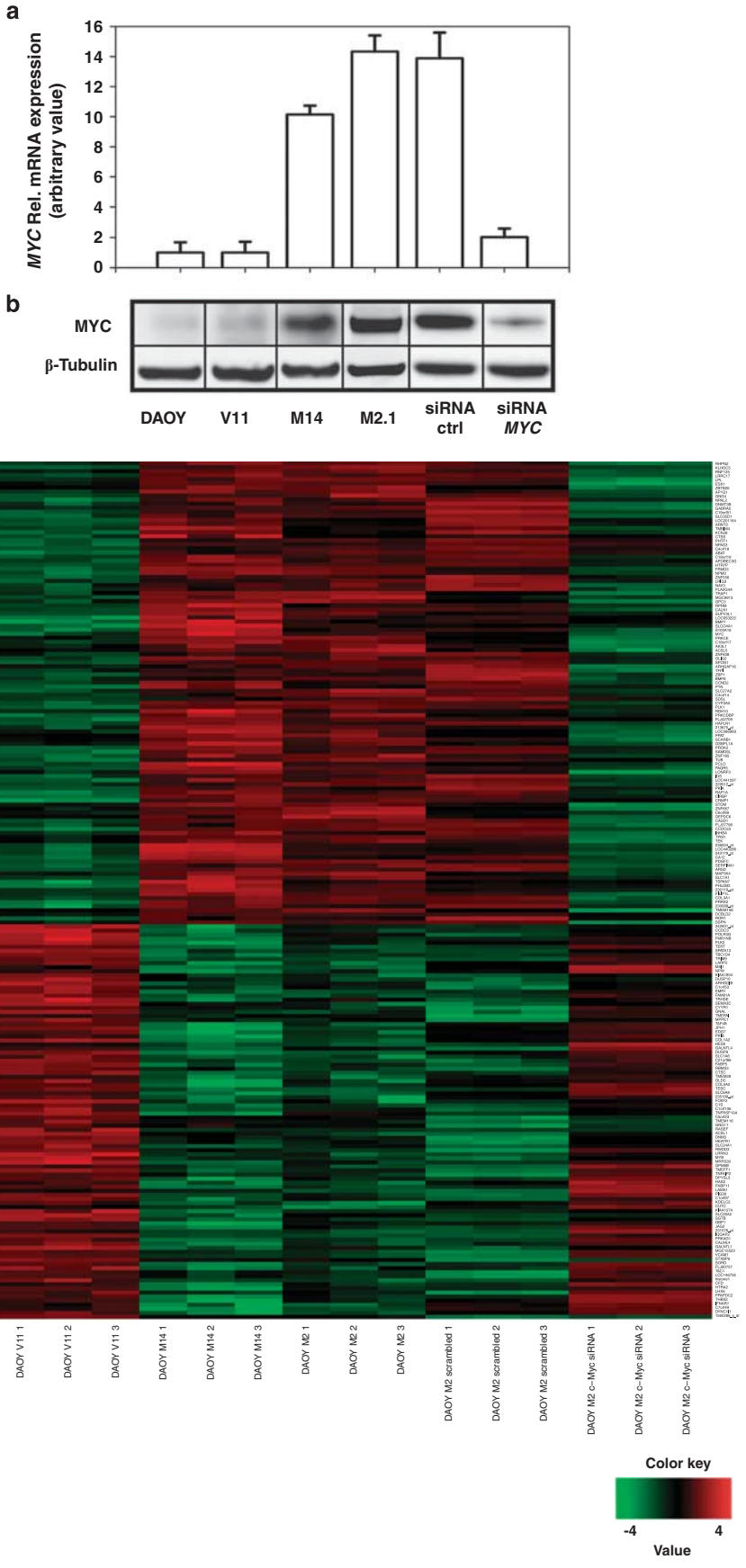
Results

Identification of MYC-responsive genes in a model of MB

To identify MYC-driven tumorigenic functions in MB, DAOY and DAOY-derived cells were genetically manipulated to either enhance or silence *MYC* expression (Stearns *et al*., 2006), and their expression profiles were analyzed by cDNA microarray. In one set of experiments, we evaluated the transcriptional changes induced by *MYC* upregulation in DAOY cells by comparing the profiles of two *MYC*-overexpressing clones (DAOY M2.1 and M14) with those of wild-type DAOY and empty-vector-transfected DAOY V11 cells. The two clones shared 98% similarity in differentially expressed genes, they were substantially identical in terms of transcriptional changes associated with *MYC* expression on the genetic background of DAOY cells. The lists of genes associated with *MYC* overexpression in DAOY M2.1 and M14 cells were intersected to select only common genes and corrected for the contribution of the empty vector. This analysis enabled us to define a list of genes associated with *MYC* overexpression. In addition, we analyzed the transcriptome of DAOY M2.1 cells transfected with *MYC*-specific small interfering RNA (siRNA) normalized to control/non-targeting siRNA and found a group of genes associated with *MYC* knockdown. In this latter set of experiments, the expression analysis was carried out at 48 h post transfection. This time point was chosen because it showed the most efficient downregulation of *MYC* and of its known direct target telomerase reverse transcriptase (*TERT*) (Supplementary Figure S1A). Figure 1 shows the variation of MYC in all cell lines in the study, at both the mRNA (Figure 1a) and protein (Figure 1b) levels.

To select only those entities up/downregulated in a consistent manner in both sets of experiments, we intersected the two lists of genes. We obtained a group of 209 genes that were responsive to *MYC* gene modulation with absolute fold change $\geqslant 2.0$ and *P*-value <0.01 (Figure 1c). Of the 209 genes, 105 were

Figure 1 Gene expression analysis. (**a**) The mRNA expression levels of *MYC* were quantified by quantitative real-time PCR in DAOY cells and in DAOY-derived cell clones (empty-vector-transfected DAOY V11, *MYC*-transfected DAOY M2.1 and M14). Data are normalized to *SDHA* and to *MYC* expression in normal human cerebellum (defined as 1). (**b**) MYC protein levels were evaluated by immunoblotting and compared with β-tubulin as the loading control (ctrl). (**c**) Heat map representing the 209 MYC-responsive genes.


a
MYC Rel. mRNA expression (arbitrary value)
16
14
12
10
8
6
4
2
0
b
MYC
β-Tubulin
DAOY
V11
M14
M2.1
siRNA ctrl
siRNA MYC
c
DAOY wt 1
DAOY wt 2
DAOY wt 3
DAOY V11 1
DAOY V11 2
DAOY V11 3
DAOY M14 1
DAOY M14 2
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DAOY M2 1
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DAOY M2 scrambled 1
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DAOY M2 scrambled 3
DAOY M2 c-Myc siRNA 1
DAOY M2 c-Myc siRNA 2
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Table 1 Differential expression of BMPs and BMPR-I/II as obtained by the cDNA microarray analysis of DAOY-derived cells

Gene name	*Description*	*M2-high MYC versus ctrl*		*M14-high MYC versus ctrl*		*MYC silenced versus ctrl*	
		FC	*P-value*	*FC*	*P-value*	*FC*	*P-value*
Ligands							
BMP2	Bone morphogenetic protein 2	1.99	0.0022	2.78	0.00056	−1.42	0.0029
BMP4	Bone morphogenetic protein 4	−1.68	0.0035	−1.17	0.33	1.61	0.00096
BMP6	Bone morphogenetic protein 6	9.40	0.0002	14.83	0.0000072	−1.71	0.00005.8
BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	2.13	0.00037	8.67	0.00004.6	−2.58	0.000001.4
Receptors							
BMPR1A	Bone morphogenetic protein receptor, type IA	−1.16	0.26	−1.11	0.72	1.47	0.00047
BMPR2	Bone morphogenetic protein receptor, type II	1.41	0.0006	−1.25	0.27	1.22	0.0095
ACVR1	Activin A receptor, type I	−1.03	0.25	1.16	0.35	2.26	0.00005.8
ACVR1B	Activin A receptor, type 2 B	−1.12	0.031	2.28	0.00072	−1.61	0.0011
ACVR2A	Activin A receptor, type IIA	−1.23	0.03	1.29	0.17	−1.57	0.00033
ACVR2B	Activin A receptor, type IIB	−1.04	0.56	1.93	0.0005	−2.15	0.00026
Pseudoreceptors							
BAMBI	BMP and activin membrane-bound inhibitor homolog	1.18	0.52	1.13	0.39	2.91	0.0000004.1
Ligand inhibitors							
GPC3	Glypican 3	2.29	0.00075	2.79	0.0042	−1.69	0.00023
Secretory antagonist							
CRIM1	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	−1.21	0.22	−1.49	0.017	1.58	0.00086

Abbreviations: BMPs, bone morphogenetic proteins; BMPR, BMP receptor; cDNA, complementary DNA; ctrl, control; FC, fold change. Ctrl, DAOY/DAOY V11; M2/M14, DAOY M2.1/M14 clones; MYC silenced, DAOY M2.1 transfected with *MYC*-small inhibitory RNA.

upregulated and 104 downregulated on ectopic expression of *MYC* (Supplementary Table 1). This group of genes was analyzed by using different bioinformatics tools, such as GeneGO MetaCore, that allowed us to draw functional networks between genes based on the literature.

Our experimental data included several known direct targets of MYC, such as *CCND2* (Adhikary and Eilers, 2005) and *TERT* (Wu *et al*., 1999), which overlapped with the MYC-target gene database (www.myccancergene.org/site/mycTargetDB.asp). In fact, overexpression of *MYC* increased the *CCND2* level by 3.1- and 2.3-fold in DAOY M2.1 and M14 cells, respectively, whereas *MYC* silencing repressed the same gene by 8.4-fold. In contrast, a gene that is known to be negatively regulated by MYC, inhibin-β A (*INHBA*), was significantly downregulated in DAOY M2.1 and in DAOY M14 cells (3.8- and 3.7-fold change, respectively) and upregulated on *MYC* silencing (3.9-fold change). The cDNA microarray data were validated by quantifying the mRNA expression level of a group of representative MYC-target genes. The expression changes of *TERT* and *CCND2* following both upregulation (Supplementary Figure S1B) and silencing of *MYC* (Supplementary Figure S1C) are reported. As a negative control, the expression of cyclin A2 (*CCNA2*), which is not a target of MYC, was analyzed (Supplementary Figure S1A,B).

Effect of MYC *modulation on components of the BMP pathway*

Growing evidence suggests the functional relevance of BMPs in the onset of a subset of MB tumors, which is currently attributed to abnormal development of GNP cells of the external granular layer of the cerebellum (Marino, 2005; Crawford *et al*., 2007; Gilbertson and Ellison, 2008; Sutter *et al*., 2010). Interestingly, several members of the BMP pathway were differentially expressed following overexpression or silencing of *MYC* (Table 1), suggesting a regulatory effect of MYC on this pathway in MB.

In particular, a general increase in the mRNA expression levels of some BMP ligands (*BMP2*, *6* and *7*) in DAOY M2.1 cells hinted at MYC-dependent activation. Moreover, subtypes of BMP receptors (BMPRs) type I-II were also represented, although only a few of them could be validated in our cellular models. Similar to the majority of BMPs, the mRNA level of glypican-3 (*GPC3*), a soluble inhibitor of BMPs (Grisaru *et al*., 2001; Filmus *et al*., 2008), increased with *MYC* upregulation, and it may be responsible for a negative-feedback loop on the pathway. Finally, our microarray data did not show significant alteration of members of the SMAD family of transcription factors or of the SMAD-target genes inhibitors of differentiation (*ID*; Miyazono, 1999). This observation would imply the involvement of alternative downstream effectors, not fully characterized yet.

BMP7 *as downstream target of MYC in MB*

The correlation analysis performed by using GeneGO set a subgroup of the selected 209 MYC-responsive genes in direct and functional links with MYC, according to published data (Supplementary Figure S2). In particular, several networks revealed a direct

correlation between *MYC* and *BMP7*, which we studied in more detail, trying to elucidate the MYC-dependent regulation of *BMP7* and the relevance of the BMP pathway in our model of MB. From the gene expression profiling data, *BMP7* was upregulated in *MYC*-overexpressing DAOY M2.1 and M14 cells (2.1- and 8.7-fold change, respectively), whereas *BMP7* was repressed in cells silenced for *MYC* (2.6-fold change; Table 1). Another BMP member, *BMP6*, was found strongly upregulated on *MYC* overexpression (9.4-fold change). However, on *MYC* silencing, the transcriptional level of *BMP6* did not consistently correlate with *MYC* levels (1.7-fold-change; Table 1).

To demonstrate that the MYC transcription factor regulates transcription of *BMP7*, the ability of MYC to bind the promoter of *BMP7* was evaluated by chromatin immunoprecipitation (ChIP), performed in different MB cell lines. Genomic DNA was extracted and precipitated with a MYC-specific antibody to enrich for MYC-binding promoter sequences, which were hybridized to a promoter oligo array (Ma *et al*., 2010). We observed an enrichment of DNA fragments surrounding the transcriptional start site of *BMP7*, including regions that contain canonical binding sites for MYC (Figure 2), demonstrating that MYC can regulate the expression of *BMP7* by binding the *BMP7* promoter.

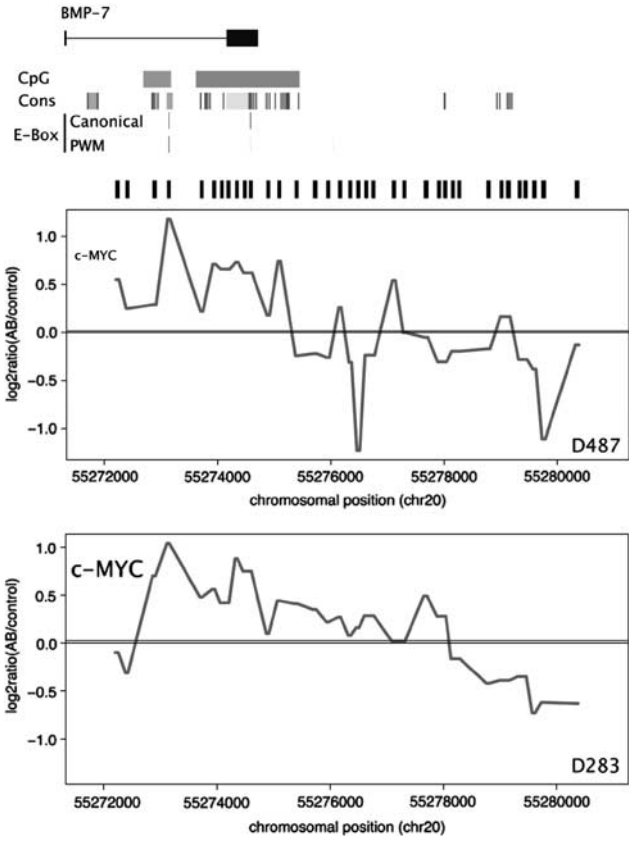

Figure 2 Binding of MYC to the *BMP7* promoter in MB cells. ChIP-on-chip data show occupancy of the *BMP7* genomic sequence by MYC in D487 and D283 medulloblastoma cells, respectively. The genomic positions for probes and their enrichment ratios are given for MYC at the *BMP7* locus in D487 and D283 cells. The horizontal line indicates the median enrichment ratio for MYC versus input as calculated from all probes for chromosome 20.

BMP7 is expressed in MB tumor samples and correlates with MYC expression

A total of 66 samples of MB available as a tissue microarray (TMA) were probed for BMP7 immunoreactivity. Cytoplasmic expression of BMP7 was quantified as positive in 10–50% cells in 17 tumors (26% MBs), as 51–90% cells in 16 tumors (24% MBs) and as >90% cells in 14 tumors (21% MBs), whereas 19 tumors (29%) expressed no BMP7 (Figure 3a and b). Notably, BMP7 could not be detected in any of the 16 normal cerebellum cores (Figure 3c). Normal human renal tubule cells were used as a positive control, as they express high levels BMP7 in the cytosol (Figure 3d).

To find a possible correlation between *BMP7* and *MYC* in primary MBs, the mRNA expression levels of both genes were determined in 38 MB samples from the TMA. The results clearly indicated a positive correlation between *BMP7* and *MYC* (Pearson's correlation value = 0.42; *P*-value = 0.0095; Figure 3e). The observation that *BMP7* and *MYC* positively correlate in primary tumors was confirmed by the unsupervised analysis of a different set of gene expression data, obtained from 46 MBs previously published by Thompson *et al*. (2006; Supplementary Figure S3). Although this analysis showed the correlation of *BMP7* with *MYC* expression in primary tumors, no significant correlation was found between *MYC* and other BMP members (from Table 1).

MYC-dependent expression of BMP7 and SMAD1/5/8 activation in MB cells

The MYC-dependent expression of BMP7 was validated in different cell lines (Figure 4). *MYC* overexpression significantly increases the mRNA level of *BMP7* (Figure 4a). On the other hand, *BMP7* was downregulated in DAOY M2.1 cells transfected with *MYC* siRNA (Figure 4b), similarly to *TERT* that is a known MYC direct target gene (see also Supplementary Figure S1A). Although *BMP6* mRNA increased on *MYC* overexpression, no effect was observed by silencing the oncogene. We could not find a significant correlation between the expression of *BMP6*, *MYC* and *TERT* (Supplementary Figure S1A).

To discriminate between the intracellular and the secreted (active) form of BMP7, both total cell lysates and supernatants were analyzed for BMP7 expression, by immunoblotting (Figure 4c) and enzyme-linked immunosorbent assay (ELISA; Figure 4d), respectively. The results confirmed that the MYC-dependent expression of BMP7 induced also an increase of the BMP7 protein, both in the precursor and the mature secreted form. In fact, BMP7 was detectable in the supernatant only in *MYC*-overexpressing cells, whereas it was undetectable (both in total cell lysates and supernatant) in cells bearing only one *MYC* copy (Figure 4c, left panel). Complementary to this effect, silencing of *MYC*

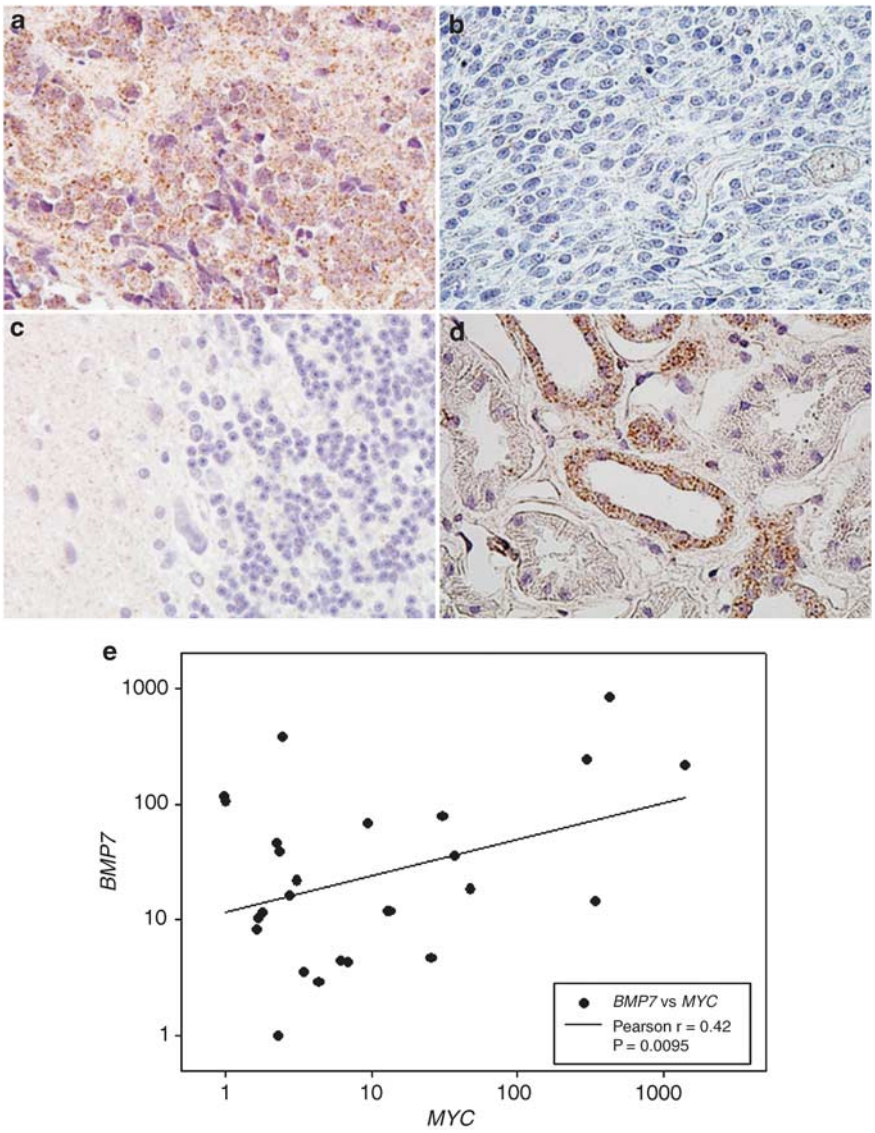

Figure 3 MB TMA analysis and *BMP7*/*MYC* correlation study in MB patients. (**a**) BMP7 immunohistochemistry showing cytoplasmic expression of BMP7 in the majority of neoplastic cells in MB (case number 97), and (**b**) no expression of BMP7 in a different MB (case number 38). (**c**) Normal cerebellum shows no BMP7 immunoreactivity. (**d**) Renal tubule cells served as a positive control showing cytoplasmic staining for BMP7. (**e**) The mRNA expression level of *BMP7* and *MYC* was evaluated by quantitative real-time PCR in a subset of the MB patient samples present on the TMA. Values were normalized to ribosomal protein 18S and to *BMP7* and *MYC* expression in normal human cerebellum (defined as 1).

caused depletion of BMP7 in cell lysates (Figure 4C, right panel) as well as in the medium (Figure 4e).

Next, we investigated whether MYC-dependent modulation of BMP7 also affected the downstream SMAD signaling pathway. Indeed, the levels of phosphorylated SMAD1/5/8 increased in MYC/BMP7-overexpressing cells, suggesting an enhanced ligand-dependent engagement of the upstream receptors (Figure 4c, left panel). On the other hand, we observed a significant reduction of SMAD1/5/8 phosphorylation in cells that were depleted of MYC (Figure 4c, right panel). As further evidence, we report on a BMP7-dependent increase of active SMAD1/5/8 in DAOY V11 and DAOY M2.1 cells, after addition of recombinant BMP7 to the medium (Figure 4f).

Targeting BMP/SMAD signaling pathway in MB

To investigate the relevance of the BMP/SMAD pathway in MB cells, we evaluated the effect of interfering with SMAD activation by using different approaches. Silencing of *BMP7* in DAOY M2.1 cells was evaluated in a time-course experiment (Figure 5a). Along with the decreased amount of secreted BMP7 in the medium (Figure 5b), BMP7 downregulation induced cell death with concomitant inhibition of cell viability (Figure 5c). These results indicate the functional relevance of BMP7 as an effector of the prosurvival functions of MYC in MB. On silencing, *BMP7* mRNA level decreased by ~80%, whereas the secreted BMP7 decreased by ~50%, compared with non-targeting-siRNA-transfected cells. This discrepancy could be explained

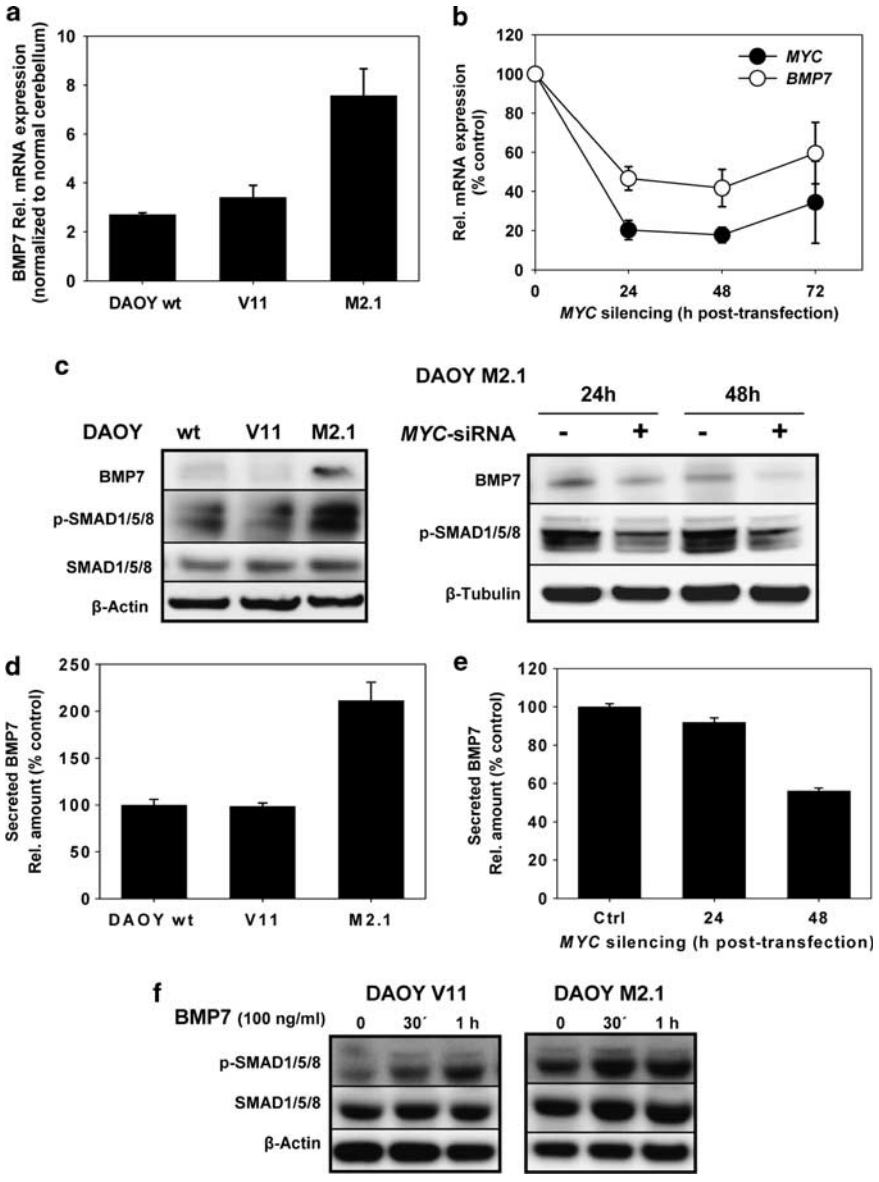

Figure 4 MYC-dependent regulation of BMP7 and SMAD activation. (**a**) *BMP7* mRNA was evaluated by quantitative real-time PCR in the indicated cell lines and reported as relative expression normalized to normal cerebellum (defined as 1). (**b**) *MYC* and *BMP7* mRNA expression levels in DAOY M2.1 cells transfected with *MYC*-siRNA are reported as the percentage of the expression in cells transfected with control/non-targeting siRNA. *SDHA* expression was used for internal normalization. (**c**) Protein levels of BMP7, phospho-SMAD1/5/8, and total SMAD1/5/8 were evaluated by western blot in DAOY cells and DAOY-derived cell clones (left panel) and in DAOY M2.1 cells with *MYC* silenced (right panel). Expression of either β-actin or β-tubulin was detected as control. (**d**, **e**) The relative amount of secreted BMP7 in cell supernatants was evaluated by ELISA following *MYC* upregulation or silencing, respectively. Percentages are in comparison to the BMP7 secreted by DAOY cells (**d**) or by DAOY M2.1 transfected with control siRNA (**e**). (**f**) The activation status of SMAD1/5/8 was also analyzed following cell stimulation with BMP7 100 ng/ml for the indicated durations.

by the documented stability of the *BMP7* mRNA (Guhaniyogi and Brewer, 2001; Buijs *et al*., 2010), which would justify a higher amount of protein detected than expected. However, we cannot exclude that the cellular responses we observed are associated not only with the downregulation of *BMP7* but also with a more general alteration of the entire BMP network following MYC modulation.

The effect of other BMPs and BMPR isoforms was evaluated by silencing *BMP2*, *BMP6* and all known *BMPR-I*/*II* receptors, which were found upregulated, together with *BMP7*, in *MYC*-overexpressing cells. Among the BMPs, downregulation of *BMP6* showed an effect similar to *BMP7* silencing (Supplementary Figure S4A), whereas among the BMPRs only downregulation of *ACVR1* reduced cell viability significantly. Although not considered as direct target of MYC, BMP6 was considered in double-knockout experiments, to prove whether silencing of both *BMP6* and *BMP7* had a more pronounced effect on cell viability than downregulating *BMP7* alone. Single silencing of *BMP6* or *BMP7* elicited similar effects on cell viability and on induction of apoptosis, as

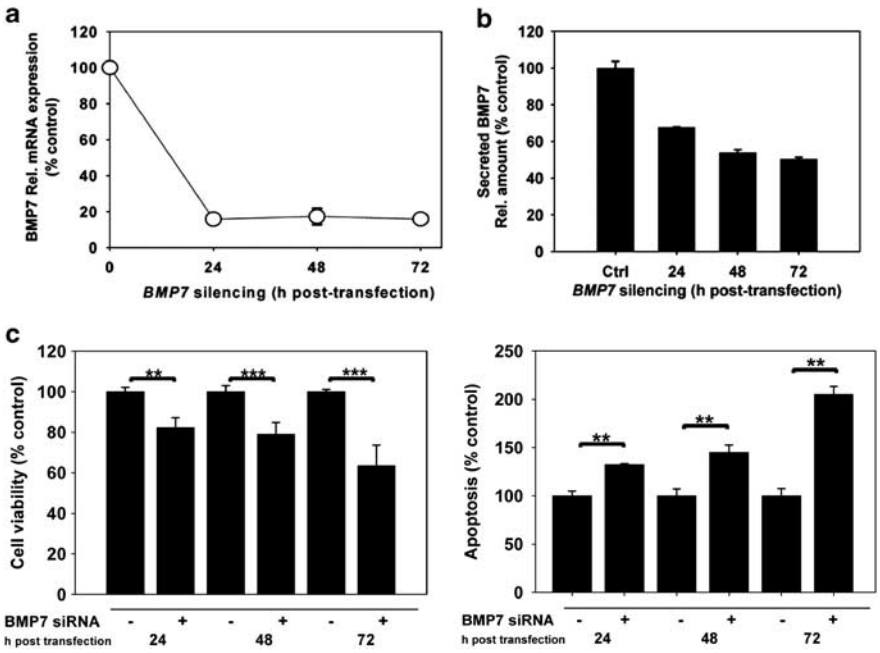

Figure 5 Effect of *BMP7* downregulation. (**a**) The mRNA expression level of *BMP7* in DAOY M2.1 cells is reported as the percentage of the *BMP7* level in control siRNA-transfected cells at the indicated time point. (**b**) Under the same conditions as in (**a**), the relative level of BMP7 secreted into the medium was evaluated by ELISA. (**c**) Cell viability and cell death induction were evaluated in DAOY M2.1 cells at the indicated time point after transfection with *MYC*-siRNA or control siRNA (**$P<0.01$, ***$P<0.001$ according to Student's *t*-test).

measured at 24–48 h after transfection, whereas silencing of *BMP6* alone was more effective after 72 h. Interestingly, the highest level of cell viability inhibition was achieved by the double knockdown (Supplementary Figure S4B), thus highlighting the relevant role of the BMP pathway for survival of MB cells.

To further study the effect of blocking the BMP/SMAD signaling pathway in MB cells, we also used dorsomorphin (DM), a small-molecule inhibitor of the BMP-dependent phosphorylation of the SMAD1/5/8 complex (Yu *et al*., 2008). MB cells were assessed for cell viability in the presence of increasing concentrations of DM. Interestingly, cells overexpressing MYC were significantly more sensitive to DM than cells bearing one *MYC* copy (Figure 6a). In the same cell lines, phosphorylation of SMAD1/5/8 was prevented by DM, confirming the inhibitory effect of the compound on the pathway (Figure 6b). Furthermore, we found a dose-dependent effect of DM on cell proliferation (Figure 6c), even though no significant difference in induction of apoptosis was found in cells overexpressing MYC, if compared with wild type and empty-vector-transfected cells (Supplementary Figure S5A). On the contrary, DM induced a significantly stronger arrest of cell proliferation in cells expressing high MYC (Figure 6c). This observation suggested that DM can function as a cytotoxic agent inducing block of cell proliferation in a MYC-dependent way.

A panel of four distinct MB-derived cell lines expressing different levels of *BMP7* was additionally considered (Supplementary Figure S5B). The results showed that high *BMP7*-expressing cells (D425 and MED1) were more sensitive to DM than cells with lower *BMP7* levels (D341 and PFSK; Figure 6d). This observation supported the presence of a BMP7-dependent mechanism sustaining cell survival, which DM is likely to target in MB cells.

Discussion

Owing to the key role in the development of MB, the oncogene *MYC* and MYC-target genes have become attractive therapeutic targets, in particular to treat the most aggressive MB subtypes (Grotzer *et al*., 2009). With the aim of identifying MYC-driven genes or pathways with potentials as therapeutic target, we profiled MB-derived cells by cDNA microarray, following genetic manipulation for either overexpressing or downregulating *MYC*. The resulting sets of differentially expressed genes were intersected to narrow down the great amount of data generated, and thus to define a group of MYC-responsive genes. These genes included known direct targets, as well as novel candidate target genes of MYC. The gene ontology analysis on the data obtained, allowed identifying pathways that are known already to have a role in onset and progression of MB, but which had not been correlated yet with MYC expression. In particular, we found several components of the BMP/SMAD pathway, known to be physiologically active during embryogenesis, promoting cell proliferation and differentiation of precursor cells of the cerebellum, from which MB arises (Thompson *et al*., 2006; Gilbertson and Ellison, 2008; Behesti and Marino, 2009; Sutter *et al*., 2010). Several *in vivo* MB models confirmed that an unbalance of proliferative/differentiation signals, due to aberrant regulation of these pathways, can induce MB tumor formation

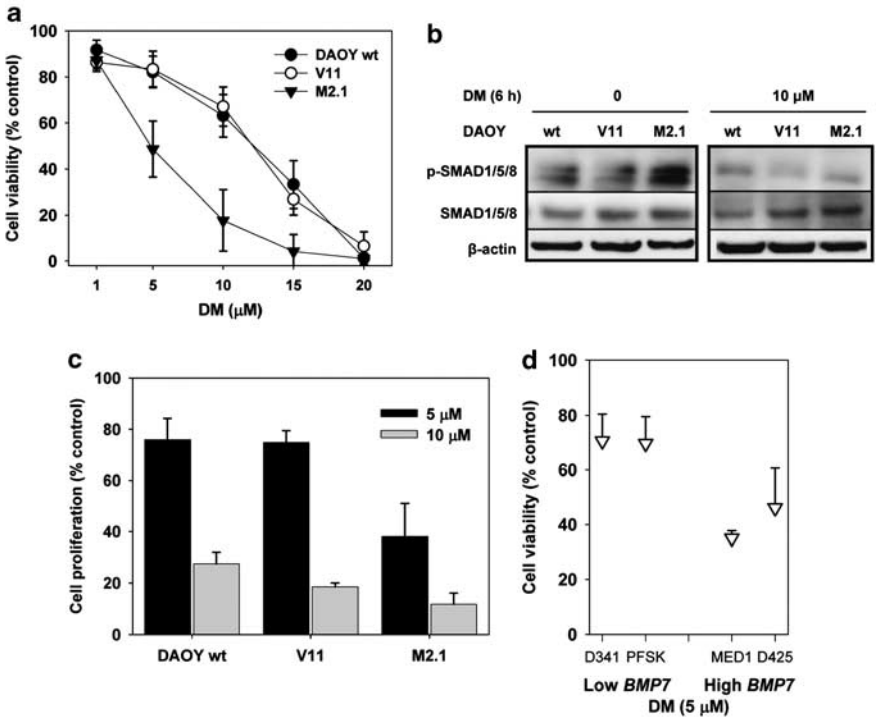

Figure 6 Inhibition of cell viability of MB cells by the BMP receptor kinase inhibitor dorsomorphin. (**a**) Cell viability was measured after 72 h of treatment with DM at the indicated concentrations. Results are expressed as the percentage of the values in vehicle-treated cells. (**b**) Phospho-SMAD1/5/8 and total SMAD1/5/8 content was investigated by western blot after treatment for 6 h with DM. (**c**) The effect of DM on cell proliferation was evaluated after 48 h. The reference was vehicle-treated cells. (**d**) The cellular response to DM was investigated in a panel of different MB-derived cell lines treated as in (**a**).

(Polkinghorn and Tarbell, 2007). In our study, *BMP7* was found consistently induced/repressed along with the up/downregulation of *MYC*, respectively, and thus chosen to be investigated in detail

Increasing evidences promote the role of the BMP/SMAD signaling pathway in determining the fate of the GNP cells of the cerebellum. In this context, components of this pathway and in particular BMP7 emerged and were further analyzed for a potential involvement in MYC-driven functions in MB. During the development of the cerebellum, BMP cytokines, including BMP7, are expressed by roof plate cells of the dorsal midline, a region adjacent to the rhombic lip (Alder *et al*., 1999), and are known to coordinate proliferation and migration of GNPs (Chizhikov *et al*., 2006). Members of the BMP family can in fact induce maturation of GNPs (Alder *et al*., 1999) and survival of postnatal granule cells (Yabe *et al*., 2002; Gilbertson and Ellison, 2008). BMP7 is constitutively expressed by cells of the choroid plexus of the forth ventricle, anatomically closed to the cerebellum (Dziegielewska *et al*., 2001; Redzic and Segal, 2004), providing a pro-proliferative sustained signal for the GNP cells (Krizhanovsky and Ben-Arie, 2006; Grimmer and Weiss, 2008). Thus, determining the correct temporal/spatial organization of cerebellar progenitor cells during early development seems to be the main role of BMP7 (Thompson *et al*., 2006). Along with this role, deregulated expression of BMP7 in the cerebellum is very likely associated with abnormal cell development and MB tumorigenesis.

As a validation of our *in vitro* results, BMP7 was analyzed by immunohistochemistry in primary MB samples. Strikingly, BMP7 was not expressed in normal cerebellum, indicating that overexpression of BMP7 in MB may contribute to the increased proliferation and survival of the tumor cells. Moreover, we found a significant correlation between the gene expression of *MYC* and *BMP7* in two distinct datasets of MB patient samples.

Further, we demonstrated that MYC can bind to the promoter of *BMP7* in different MB cell lines by using a ChIP-on-chip approach. That *BMP7* is a direct target of MYC was previously reported by Zeller *et al*. (2006) who performed a global mapping of MYC-binding genomic regions in a model of human B-lymphoid tumor. *BMP4*, another member of the same family, was described to be a target of MYC in blood cells (Fernandez *et al*., 2003). These two reports represent the only experimental evidences, to our knowledge, about a transcriptional control of the BMP pathway by MYC, lacking however of functional information. More specifically, no previous studies have mentioned MYC-driven regulation of this pathway as a significant molecular network in MB.

This investigation, therefore, suggests for the first time the existence of a functional link between the overexpression of *MYC* and abnormal regulation of the BMP pathway in a model of MB. Furthermore, the functional relevance of the BMP-dependent signaling pathway in MB was evaluated by using DM,

a small-molecule inhibitor preventing the activation of SMAD1/5/8 by interfering with the kinase activity of the receptors BMPR-I/II (Anderson and Darshan, 2008; Yu *et al*., 2008). Conversely, DM has no, or very limited, effect on other components of the transforming growth factor-β family (fox example, transforming growth factor-β1 and activin A; Anderson and Darshan, 2008). We reported here the ability of DM to strongly impair proliferation and cell viability of MB-derived cells. More interestingly, cells expressing low MYC levels were significantly less sensitive to DM than cells overexpressing the oncogene, thus indicating that the compound can affect a MYC-driven pro-survival response. Moreover, silencing of *BMP7* induced cell death and inhibition of cell viability, although it was not as efficient as DM. This observation, however, is reasonable, because of the ability of the compound to block the pathway by interfering with more components at the same time.

Our results show the feasibility and potential benefit of a selective block of the BMP/SMAD pathway, by either silencing *BMP7* or using small-molecule inhibitors. A therapeutic approach interfering with activation of the BMP pathway was investigated here, for the first time, in MB cells and would represent one attractive strategy, to be considered in combination with targeting of sonic hedgehog and Wnt pathways (Chen *et al*., 2002; Romer and Curran, 2005; Luo *et al*., 2007; Rossi *et al*., 2008). Additional investigations on the role of other BMP family members will emphasize the relevance of the whole pathway in the development of MB. They will also help to shed lights on the emerging recent data showing opposite effects of BMPs, such as BMP2 and BMP4, to whom either anti-apoptotic (Iantosca *et al*., 1999) or anti-proliferative properties (Zhao *et al*., 2008) were assigned.

In conclusion, our results provide strong evidence of the induction of *BMP7* as a MYC-dependent mechanism and suggest BMP7 as an effector regulatory molecule, in the context of MYC-driven neoplastic transformation of MB precursor cells. This study, not only contributes to a better understanding of the prosurvival functions of MYC in MB, but it also suggests a rationale for targeting the BMP pathway in MB patients with *MYC* amplification/overexpression.

Materials and methods

MB cell lines

The human MB-derived DAOY, D341, D425, MED-1 and PFSK cell lines used in this study were obtained and maintained in culture as previously described (von Bueren *et al*., 2007). The stable clones DAOY V11 (empty vector transfected), DAOY M2.1 and DAOY M14 (*MYC* vector transfected) were maintained in selective medium in the presence of 500 μg/ml G418 (Stearns *et al*., 2006).

RNA interference

The cells (70–80% confluent) were transfected using either SMARTpool siRNA specific for *MYC* or siCONTROL Non-targeting siRNA Pool as a control (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA). Each pool of siRNA was used at the final concentration of 50 nM in combination with Dharmafect 4 transfection reagent (Dharmacon), according to the manufacturer's instructions. Cells were harvested for both mRNA and protein extraction, to assess gene expression by quantitative real-time PCR and protein content by immunoblotting. To selectively target *BMP7* and other components of the BMP/SMAD pathway (*BMP2*, *BMP6* and BMP receptors *BMPR1A*, *BMPR1B*, *BMPR2*, *ACVR1*, *ACVR1B*, *ACVR1C*, *ACVR2A*, *ACVR2B* and *ACVRL1*), Ambion Silencer select predesigned siRNAs (Ambion Diagnostics, Applied Biosystems, Foster City, CA, USA) were used at 20 nM final concentration. As control, Silencer Negative Control siRNA #1 (cat. AM4611; Ambion Diagnostics) was used under the same conditions.

DNA microarray expression profiling and data analysis

The cDNA microarray analysis was performed in collaboration with the Functional Genomic Center of the University Zurich. Gene expression data were obtained by hybridizing Human Genome-U133 Plus 2.0 Affymetrix GeneChips arrays (Affymetrix, Santa Clara, CA, USA), on which $>47\,000$ transcripts were represented. Raw data generated by the GCOS Software (Affymetrix) were processed by using the RMA method (Irizarry *et al*., 2003) and further statistically analyzed by using the software R and applying Student's *t*-test. Each experiment represented a group of three independent biological replicates. A gene was considered expressed only if the average normalized signal in at least one of the two groups compared was above 25. Results are expressed as fold change, and differences in expression were considered significant if fold change $\geqslant 2.0$ and *P*-value <0.01. The expression data were deposited in NCBI's Gene Expression Omnibus (Edgar *et al*., 2002) and are accessible through GEO series accession number GSE22139 (http://www.ncbi.nlm.nih.gov/geo). Cluster analysis of the data was performed by using the heatmap.2 method of the 'gplots' package (R Development Core Team, 2008). The GeneGO MetaCore (GeneGO, St Joseph, MI, USA) was used to define functional annotations for the selected genes, thus assigning them to ontological categories for association with relevant biological processes and pathways.

MB TMA and BMP7/MYC correlation study in MB patient samples

The tumor material used to create the MB TMA originates from three sets of archival MB samples, diagnosed in Zurich, Switzerland ($n=46$), Cairo, Egypt ($n=14$) and Tübingen, Germany ($n=6$). All these tumor samples were reviewed independently by two neuropathologists and diagnosed as MB.

TMA paraffin sections were deparaffinized in xylene and rehydrated stepwise with ethanol. The immunohistochemistry determination of BMP7 was carried out using the catalyzed signal amplification II system (Dako, Glostrup, Denmark) according to the manufacturer's protocol. The endogenous peroxidase activity was blocked incubating the specimens with 0.3% H_2O_2 for 5 min, followed by 5 min with a protein-based blocking reagent and incubation with a BMP7-specific antibody (Abcam, Cambridge, UK; 1:3000), for 30 min at room temperature. The signal was detected in the presence of diaminobenzidine. Immunoreactivity was scored as (i) −, if $<10\%$; (ii) +, if 10–50%; (iii) ++, if 51–90%; (iv) +++, if $>90\%$ of neoplastic cells with cytoplasmic staining for BMP7. Human normal kidney tissues on paraffin sections served as a positive control. From a subset of 38 samples from

the MB patients diagnosed in Zurich (Switzerland) sufficient tumor material was available to perform quantitative real-time PCR for the analysis of *BMP7* and *MYC* mRNA expression. Total RNA was isolated from formalin-fixed paraffin-embedded tumor tissue as described previously (Kunz *et al*., 2006) by using the Optimum formalin-fixed paraffin-embedded for paraffin block RNA isolation kit (Ambion Diagnostics). Paraffin from 1×20 to $2 \times 20\,\mu m$ slices of formalin-fixed paraffin-embedded MB samples was removed by washing the samples with xylene for 30 min. Samples were collected by centrifugation, washed with ethanol,and allowed to air dry at room temperature prior final centrifugation. Samples were then resuspended in 10 µl (60 U/µl) proteinase K and 100 µl of digestion buffer, and incubated at 37°C for 3 h and then at room temperature for further 12 h. After complete digestion, RNA was extracted according to the manufacturer's protocol.

To validate our results, expression data obtained from a different group of primary MB tumors (Thompson *et al*., 2006) was used to perform an unsupervised analysis on the mRNA expression of *BMP7* and *MYC*.

Cell viability, cell proliferation and apoptosis assays

Cell viability was evaluated using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) based on the reactivity of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Alternatively, the number of viable cells was determined by trypan blue exclusion using a hemocytometer. Proliferation was quantified by the chemiluminescence-based Cell Proliferation ELISA BrdU (F Hoffmann-La Roche AG, Basel Switzerland). Activation of caspases 3 and 7 was detected by using the Caspase-Glo 3/7 Assay (Promega Corporation); hystone-associated DNA fragments were quantified by Cell Death Detection $ELISA^{PLUS}$ assay (Roche Diagnostics). Data are expressed as average values from three independent experiments.

Gene expression analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions. After enzymatic digestion of DNA with RNase-free DNase (Qiagen), 0.5–1 µg of total RNA was used as template for reverse transcription, which was triggered by random hexamer primers and performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed under conditions optimized for the ABI7900HT instrument, using Gene Expression Master Mix (Applied Biosystems). Probe-primer specific for the following genes (purchased from Applied Biosystems) were used: *MYC* (Hs00153408_m1), *BMP7* (Hs00233476_m1), *TERT* (Hs00162669_m1), *CCND2* (Hs00277041_m1) and *CCNA2* (Hs00153138_m1). Normal human cerebellum was used as a reference (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). The relative gene expression was calculated for each gene of interest by using the $\Delta\Delta C_T$ method, where cycle threshold (C_T) values were normalized to the housekeeping genes succinate dehydrogenase complex subunit A (*SDHA*) (Hs00188166_m1) and β-actin (Hs99999903_m1).

Western blot analysis and ELISA

Total protein extracts were obtained from $0.5–1.5 \times 10^6$ cells lysed with RIPA buffer (50 mM Tris-Cl, pH 6.8, 100 mM NaCl, 1% Triton X-100, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor Cocktail (Roche Applied Sciences) and with the phosphatase inhibitors β-glycerophosphate (20 mM) and Na_3VO_4 (200 µM). Proteins were resolved by SDS–polyacrylamide gel electrophoresis and western blotting on polyvinylidene fluoride membranes (Amersham, GE Healthcare, UK). After binding of the indicated antibodies, the signal was detected by chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, IL, USA). Antibodies specific for BMP7 (4E7) and SMAD 1/5/8 (N-18) were purchased from Santa Cruz Biotechnology Inc., whereas antibodies against phospho-SMAD1 (Ser463/465), SMAD5 (Ser463/465), SMAD8 (Ser426/428) and MYC were from Cell Signaling Technology, Inc. (Danvers, MA, USA). As loading controls, β-tubulin and β-actin (Sigma-Aldrich Chemie GmbH, Buchs, Germany) were detected. According to the experimental setting, cells were incubated in the presence of human recombinant BMP7 (Calbiochem-Merck, Darmstadt, Germany) for 0.5 or 1 h, and cell lysates were analyzed by western blotting. To quantify secreted BMP7 in cell supernatants (100 µl from each sample), the human BMP7 ELISA kit (RayBiotech Inc., Norcross, GA, USA) was used following the manufacturers' instructions.

ChIP-on-chip

ChIP-on-chip experiments using a MYC antibody were performed as previously described (Ma *et al*., 2010). The MB-derived cell lines D487 and D283 were used in this study.

Statistical analysis

All experiments were performed at least in triplicates. Data are represented as mean ± s.d. For *in vitro* experiments, the Student's *t*-test was used. *P*-values of <0.01 were considered significant. Pearson's correlation test and Fisher's exact test were used for *in vivo* gene correlation.

Conflict of interest

The authors declare no conflict of interest.

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Therapeutic Discovery

Molecular Cancer Therapeutics

The Quassinoid Derivative NBT-272 Targets Both the AKT and ERK Signaling Pathways in Embryonal Tumors

Deborah Castelletti[1], Giulio Fiaschetti[1], Valeria Di Dato[2], Urs Ziegler[3], Candy Kumps[4], Katleen De Preter[4], Massimo Zollo[2], Frank Speleman[4], Tarek Shalaby[1], Daniela De Martino[2], Thorsten Berg[5], Angelika Eggert[6], Alexandre Arcaro[7], and Michael A. Grotzer[1]

Abstract

The quassinoid analogue NBT-272 has been reported to inhibit MYC, thus warranting a further effort 7to better understand its preclinical properties in models of embryonal tumors (ET), a family of childhood malignancies sharing relevant biological and genetic features such as deregulated expression of MYC oncogenes. In our study, NBT-272 displayed a strong antiproliferative activity *in vitro* that resulted from the combination of diverse biological effects, ranging from G_1/S arrest of the cell cycle to apoptosis and autophagy. The compound prevented the full activation of both eukaryotic translation initiation factor 4E (eIF4E) and its binding protein 4EBP-1, regulating cap-dependent protein translation. Interestingly, all responses induced by NBT-272 in ET could be attributed to interference with 2 main proproliferative signaling pathways, that is, the AKT and the MEK/extracellular signal-regulated kinase pathways. These findings also suggested that the depleting effect of NBT-272 on MYC protein expression occurred via indirect mechanisms, rather than selective inhibition. Finally, the ability of NBT-272 to arrest tumor growth in a xenograft model of neuroblastoma plays a role in the strong antitumor activity of this compound, both *in vitro* and *in vivo*, with its potential to target cell-survival pathways that are relevant for the development and progression of ET. *Mol Cancer Ther; 9(12); 3145–57. ©2010 AACR.*

Introduction

Embryonal tumors (ET) represent an important group of childhood malignancies arising from different tissues of fetal origin (1, 2). Medulloblastoma (MB) is a tumor of the central nervous system, originating from granular progenitor cells of the cerebellum (3) and accounting for about 20% of all childhood brain tumors (4). Neuroblastoma (NB) is the most common extracranial solid cancer in children originating from the sympathetic nervous system (5, 6). Constant progress has been achieved in clinical practice by increasing the overall survival rate of MB and NB patients, although novel therapeutic strategies are still needed, particularly to treat the high-risk patients characterized by a more aggressive disease and poor prognosis. In both MB and NB, high-grade malignancy is often associated with overexpression or amplification of MYC oncogenes (7–11) playing a role in embryogenesis and, if aberrantly expressed, in tumor development as well (12).

There is a long history of therapeutic benefits produced by natural compounds and some of these have formed the backbone for further targeted chemical modifications improving efficacy and selectivity. Quassinoids have been investigated for their antineoplastic properties (13), which has led to the identification of interesting candidates such as bruceantin, with significant inhibitory effects on leukemia, lymphoma, and myeloma cell growth, both *in vitro* and *in vivo* (14). Bruceantin was reported to induce apoptosis and cell differentiation, thus justifying efforts to develop quassinoid analogues with improved efficacy.

Among the derivatives of bruceantin, NBT-272 displayed a significantly stronger toxicity than the parent molecule, as tested in different models of hematological and solid tumors (15, 16). Moreover, NBT-272 was reported to induce downregulation of c-MYC in MB-derived cells (16), thus raising the question of the

Authors' Affiliations: [1]Department of Oncology, University Children's Hospital of Zurich, Switzerland; [2]Department of Biochemistry and Medical Biotechnology, University of Naples, Italy; [3]Center for Microscopy and Image Analysis University of Zurich, Switzerland; [4]Center for Medical Genetics Ghent, Belgium; [5]Institute for Organic Chemistry, University of Leipzig, Germany; [6]Department of Oncology, University Children's Hospital of Essen, Germany; and [7]Division of Pediatric Hematology/Oncology, Department of Clinical Research, University of Bern, Switzerland

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Corresponding Author: Michael A. Grotzer, University Children's Hospital of Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. Phone: 41-44-266-7111; Fax: 41-44-266-7171. Email: Michael.Grotzer@kispi.unizh.ch

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mechanisms underlying NBT-272's cytotoxicity and its selectivity for MYC in ET.

Besides activating programmed cell death, quassinoids were shown to arrest cell growth by impairing protein synthesis mediated by the eukaryotic translation initiation factor 4E (eIF4E; ref. 17). The phosphorylation status of eIF4E plays an important biological role in modulation of normal cell growth in response to extracellular stimuli and during development (18). However, eIF4E was also reported to be an oncogene (19) and its transformation properties were attributed, at least in part, to induction of cell cycle progression through cyclin D1 (CCND1) and MYC (20). Activation of eIF4E itself is mainly regulated by 2 pathways, namely the mitogen-activated protein kinase pathway (21) and the AKT/mammalian target of rapamycin (mTOR) pathway via the 4E binding protein (4EBP-1; ref. 22).

In this study, we have investigated the effect of NBT-272 in different cellular models of ET, both *in vitro* and *in vivo*. The compound triggered multiple cellular responses, ranging from cell cycle arrest to induction of autophagy, which are downstream effector mechanisms of the AKT/mTOR and extracellular signal-regulated kinase (ERK) pathways. In turn, interference with these signaling events could also explain the inhibitory effect of NBT-272 on protein synthesis and on expression of c-MYC/MYCN in different ET entities.

Materials and Methods

Cell lines

The following human cell lines were used: the NB SK-N-BE, SH-SY5Y, and WAC-2 (23; G. M. Brodeur, Children's Hospital of Philadelphia, Philadelphia, PA); retinoblastoma (RB) Y79 and WERI (A. Eggert, University Children's Hospital, Essen, Germany); Ewing's sarcoma family of tumors (ESFT) A673 and TC71 (H. Kovar, St. Anna Children's Cancer Research Institute, Vienna, Austria); and malignant rhabdoid tumor (MRT) LP and MON (O. Delattre, Institut Curie, Paris, France). MB DAOY and D341 were purchased from American Type Culture Collection. Cells that were not purchased from the American Type Culture Collection were authenticated by comparative genomic hybridization (in the laboratories mentioned above or as described below). The clone DAOY M2 (overexpressing c-MYC) was kindly provided by D. Stearns (Johns Hopkins University, Baltimore, MD; ref. 11). NB, ESFT, and MRT cell lines were grown in RPMI medium (Life Technologies/Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) and penicillin/streptomycin (PS)/L-glutamine, whereas RB cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 15% (v/v) FCS Gold (Invitrogen), 10 μg/mL insulin (Sigma-Aldrich), 50 μmol/L 2-β-mercaptoethanol (Sigma-Aldrich), and PS/L-glutamine. MB cells were cultured in Richter's zinc option medium (Invitrogen) supplemented with 10% (v/v) FCS and PS/L-glutamine.

Array CGH

The ET-derived cell lines used in the study were profiled by array comparative genomic hybridization (aCGH) using a custom 60K Embryonal Tumor array CGH-chip (ET-aCGH chip; Agilent Technologies), as described by Kumps et al. (24).

Cell viability, clonogenic, and apoptosis assays

The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)-based CellTiter 96 AQueous One assay (Promega) was used to quantify cell viability. Colony formation in agar was tested by seeding single cells (4,000 per well) in agar-containing medium [0.35% (w/v) final concentration] on top of a 0.5% (w/v) agar layer. Apoptosis was investigated by quantifying the activation of caspases, by using the Caspase-Glo 3/7 Assay (Promega) according to manufacturer's instructions. The In Situ Cell Death Detection Kit (Roche) was also employed to evaluate the proapoptotic response of ET cells. The terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction was done in the presence of fluorescein-labeled nucleotides and followed by detection under a fluorescent microscope (Axioskop 2; Zeiss).

Cell cycle analysis

Cells [$(5–10) \times 10^5$] were treated for the indicated time intervals with different doses of NBT-272 or were mock treated (control). After washing with PBS, the cells were washed and harvested in a 2 mmol/L EDTA/PBS solution. Cell pellets were fixed with ice-cold 70% ethanol on ice for 40 to 60 minutes and, after centrifugation at 2,500 *g* for 5 minutes, resuspended with 50 μg/mL propidium iodide/PBS containing 100 U/mL RNAase (Sigma-Aldrich). The DNA content was estimated by using a BD FACS Canto II flow cytometry system for acquisition and the FlowJo software for the data analysis.

Gene expression analysis by PCR arrays

Total RNA from DAOY, D341, SK-N-BE, and SH-SY-5Y cells, treated with 20 nmol/L NBT-272 for 24 hours or left untreated, was subjected to reverse transcriptase (RT; RT^2 First Strand Kit; SABiosciences). The resulting cDNA was used as a template for RT^2 Profiler PCR Array (SABiosciences) for pathway-focused gene expression analysis (using sets of arrayed TaqMan probes for cell cycle and apoptosis genes). Gene expression was expressed as $\log_2$ ratio (where ratio is $2^{-\Delta\Delta C_t}$), relative to normalization to untreated control samples and to the average expression of five housekeeping genes (B2M, HPRT1, RPL13A, GAPDH, and ACTB). Only statistically significant gene expression changes over 3 independent experiments were considered.

Western blot analysis

Total cell lysates were prepared in a radioimmunoprecipitation assay buffer buffer [50 mmol/L Tris-HCl (pH 7.5), 0.1% sodium laurilsulfate, 0.1% sodium deoxycholate, 150 mmol/L NaCl, 1% NP40, 1 mmol/L EDTA, and 1 mmol/L EGTA], supplemented with protease inhibitors (Roche) and phosphatase inhibitors (10 mmol/L sodium fluoride, 10 mmol/L sodium orthovanadate, and 10 mmol/L sodium β-glycerophosphate). The amount of protein was determined by BCA Protein Assay (Pierce). Cell lysates in SDS-sample buffer were boiled for 8 minutes and equal protein amounts were resolved by 4% to 12% gradient SDS-PAGE and immunoblotting using polyvinylidene difluoride membranes. The antibodies reacting against the following proteins were employed: CCND1, caspase-3, AKT, mTOR, phospho-ERK 1/2(Tyr202/204; Santa Cruz Biotechnology); ERK, phospho-AKT (Ser473), phospho-MEK1/2 (Ser217/221), phospho-RB (Ser795), RB, phospho-S6 (Ser235/236), S6, phospho-mTOR (Ser2248), phospho-MNK1 (Thr197/202), MNK1, phospho-eIF4E (Ser209), eIF4E, 4EBP-1, phospho-4EBP-1 (Thr37/46), cyclin E, CDK2, CDK4, PARP, LC-3, c-MYC, MYCN, and ATG5 (Cell Signaling); β-tubulin and β-actin antibodies were purchased from Sigma-Aldrich, whereas the anti-SQSTM1/p62 was purchased from BIOMOL International. To inhibit proteasome-dependent protein degradation, cells were treated with 50 μmol/L MG-132 (Sigma-Aldrich). The PI3K/mTOR inhibitor BEZ-235 was purchased by Axon Medchem.

Transmission electron microscopy

After treatment (with NBT-272, rapamycin, or vehicle) for different time intervals, DAOY and SK-N-BE cells were subjected to fixation at room temperature for 30 minutes with 2% glutaraldehyde and 0.8% formaldehyde in 50 mmol/L sodium cacodylate (pH 7.3), followed by 30-minute exposure to 1% OsO_4 and 1.5% $K_4Fe(CN)_6$ in 50 mmol/L sodium cacodylate (pH 7.3). Fixed samples were contrasted using 2% uranyl acetate in water for 2 hours, progressively dehydrated in increasing concentrations of ethanol, and embedded into epon (Catalys). Ultrathin sections of 50 nm were prepared and contrasted with uranyl acetate and lead citrate. Samples were analyzed by using a CM 100 transmission electron microscope (TEM; Philips).

In vivo xenograft model

Human NB SH-SY5Y cells were stably transfected with a pLentiV5-Luciferase-expressing vector (Invitrogen) as described elsewhere (25). SH-SY5Y-LUC clones were grown under standard conditions in DMEM containing 10% (v/v) FCS, L-glutamine, and blasticidine for selection.

Eight-week-old female athymic nude mice from Harlan Laboratories were inoculated subcutaneously in the right rear flank with 2×10^6 SHSY5Y-LUC cells, injected in 100 μL PBS solution. Tumor cell growth was monitored weekly by measuring luminescence emission, referred to as bioluminescence imaging (BLI) levels, using the IVIS 3D Illumina Imaging System (Xenogen Corp.). A detailed procedure can be obtained from Caliper. The BLI/tumor size ratio was quantified by encircling the luciferase-emitting areas using the Living Images Software 3.2 (Xenogen). Three weeks after implantation, the mice were divided into 2 homogeneous groups based on their BLI level (photon/s/cm^2). Each group received either 10 μmol/L per week of NBT-272 or vehicle (EtOH 0.003%/PBS), administered as 100 μL solution each time, 3 times a week. The analysis of the tumor growth was done 3, 4, and 6 weeks after the first NBT-272 administration. The dose was adjusted by taking into account preliminary data provided by NaPro BioTherapeutics, Inc. (maximum tolerated dose = 0.9 mg/kg/wk; ref. 15) and the *in vitro* results. The treatment was done for 6 weeks before the animals were sacrificed, and tumor samples were analyzed by immunohistochemistry and Western blotting. Quantitative data analysis of the tumor size was done by ANOVA statistical tests, using the Statview program.

All animal experiments were conducted according to national and international guidelines and following approval by the Institutional Animal Care and Ethical Committee of CEINGE-University of Naples Federico II (Protocol 29, September 30, 2009) and of the Italian Ministry of Health (Dipartimento Sanità Pubblica Veterinaria D.L. 116/92).

Immunohistochemistry

Paraffin-embedded tumor samples were treated with xylene, heated for 45 minutes (in citrate buffer, pH 6.0), and finally treated for 15 minutes with 1% H_2O_2 (Carlo Erba Reagents). Tissues for immunohistochemistry were blocked with 3% bovine serum albumin, 5% total goat serum, and 0.3% Tween 20/PBS (1 hour, room temperature). Primary antibodies reactive against human Ki67 (M7240; Dako, Glostrup, Denmark) and human cleaved caspase-3 (9661; Cell Signaling) were used (1 hour, room temperature, diluted 1:100 in blocking solution). After incubation with a biotin-conjugated secondary antibody (1 hour, room temperature), the detection was done in the presence of 3,3′-diaminobenzidin (Dako) according to the manufacturers' protocol. The sections were counterstained by hematoxylin/eosin (Bio Optica), washing 3 times with 0.2% Triton X-100/PBS after every incubation steps. As negative control, blocking solution was used instead of the primary antibody.

Results

NBT-272 strongly impaired cell growth in ET-derived cell lines

NBT-272 (NaPro BioTherapeutics, Inc.; Fig. 1A) is a semisynthetic analogue of bruceantin (14, 17), with 10-fold higher toxicity than the parent compound *in vitro* (15). Previously published data showed the ability of NBT-272

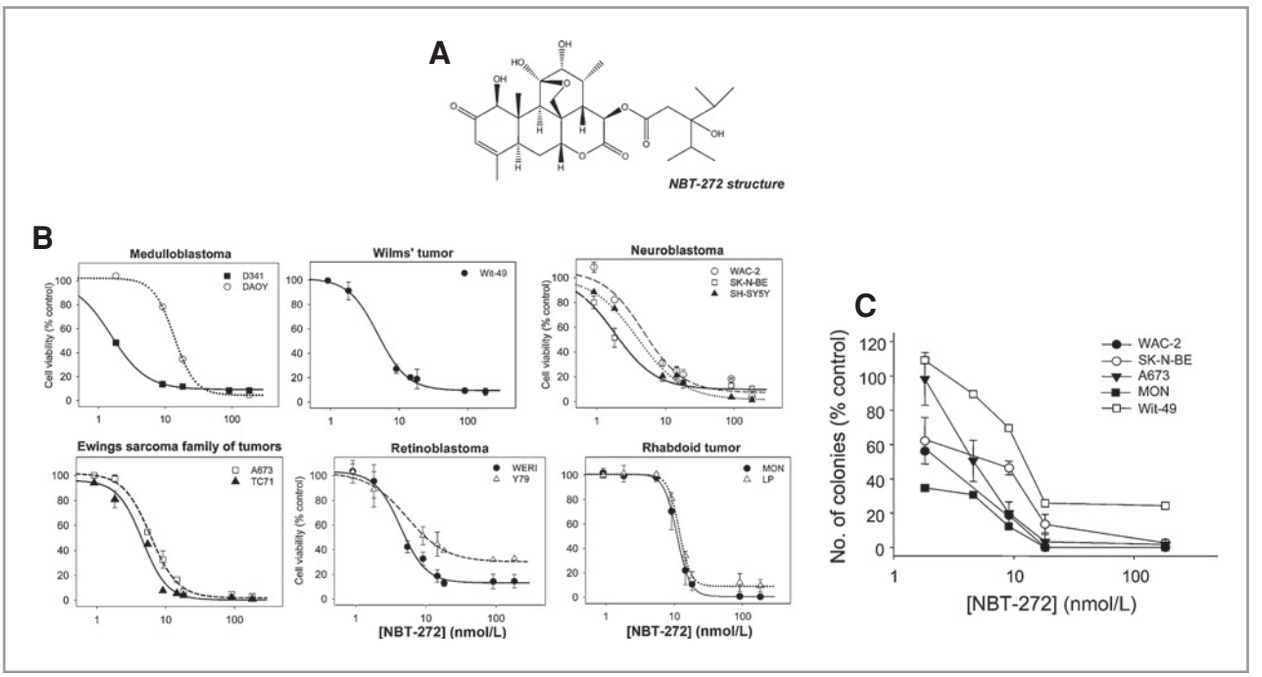

Figure 1. Dose-dependent effect of NBT-272 on cell viability and colony formation in ET-derived cell lines. A, chemical structure of NBT-272. B, MTS assays were done 72 hours after addition of increasing concentrations of NBT-272 (1–182 nmol/L) in 13 ET-derived cell lines. C, five cell lines were evaluated for anchorage-independent growth in soft agar. NBT-272 reduced colony formation by 50% relative to monolayer cultures at concentrations lower than/similar to IC_{50} values. IC_{50} (nmol/L): WAC-2, 2.3; SK-N-BE, 4.3; A673, 4.6; MON, < 1; Wit-49, 9.7.

to drastically reduce the protein expression level of the oncogene c-MYC in MB-derived cell lines (16), although the exact mechanism of action remained unexplored. In this study, the biological responses induced by NBT-272 were investigated in a panel of 13 cell lines, representative of 6 different ET entities (Fig. 1). Expression of c-MYC and MYCN was evaluated in each cell line both at protein (Supplementary Fig. S1A) and mRNA levels (Supplementary Fig. S1B); these varied significantly, reflecting the gene expression ranging from single copy to gene amplification.

The ET cell lines were profiled for copy number changes (gain/loss) using aCGH. In Supplementary Fig. S2, the aCGH data of 8 cell lines are reported. Some cell lines displayed very few genomic aberrations (1q gain in the MRT cells LP, and 1q gain and 8q gain in MB D341), whereas others have very complex profiles, such as WERI cells with a 1q gain region that appeared very discontinuous.

The effect of NBT-272 was first evaluated in terms of cell viability, clearly showing a very potent effect of the compound in all selected ET-derived cell lines (Fig. 1B; Table 1). The IC_{50} values, calculated from the dose–response curves, varied in the nmol/L range of concentration, that is, about 2 orders of magnitude lower compared with many small-molecule inhibitors investigated as anticancer drugs. Additionally, 5 representative ET cell lines (WAC-2, SK-N-BE, A673, MON, Wit-49) were tested for anchorage-independent growth in soft agar in the presence of NBT-272 (Fig. 1C). The compound reduced colony formation by 50% (compared with mock-treated controls) at doses that were similar or lower than the IC_{50} obtained in cell viability assays (Table 1).

Table 1. The IC_{50} values relative to NBT-272 toxicity, as calculated from the curves fitting the data in Figure 1B

ET entity	Cell line	[NBT-272] IC_{50}(nmol/L)
Medulloblastoma (MB)	DAOY	13.6
	DAOY M2	4.9
	D341	1.5
Neuroblastoma (NB)	WAC-2	4.8
	SK-N-BE	1.8
	SH-SY5Y	3.5
Retinoblastoma (RB)	WERI	4.4
	Y79	5.4
Wilms' tumor (WT)	Wit-49	4.9
Ewing sarcoma family of tumors (ESFT)	A673	6.0
	TC71	4.6
Malignant rhabdoid tumor (MRT)	MON	11.8
	LP	11.0

Note: DAOY M2 cells (overexpressing c-MYC) are also reported.

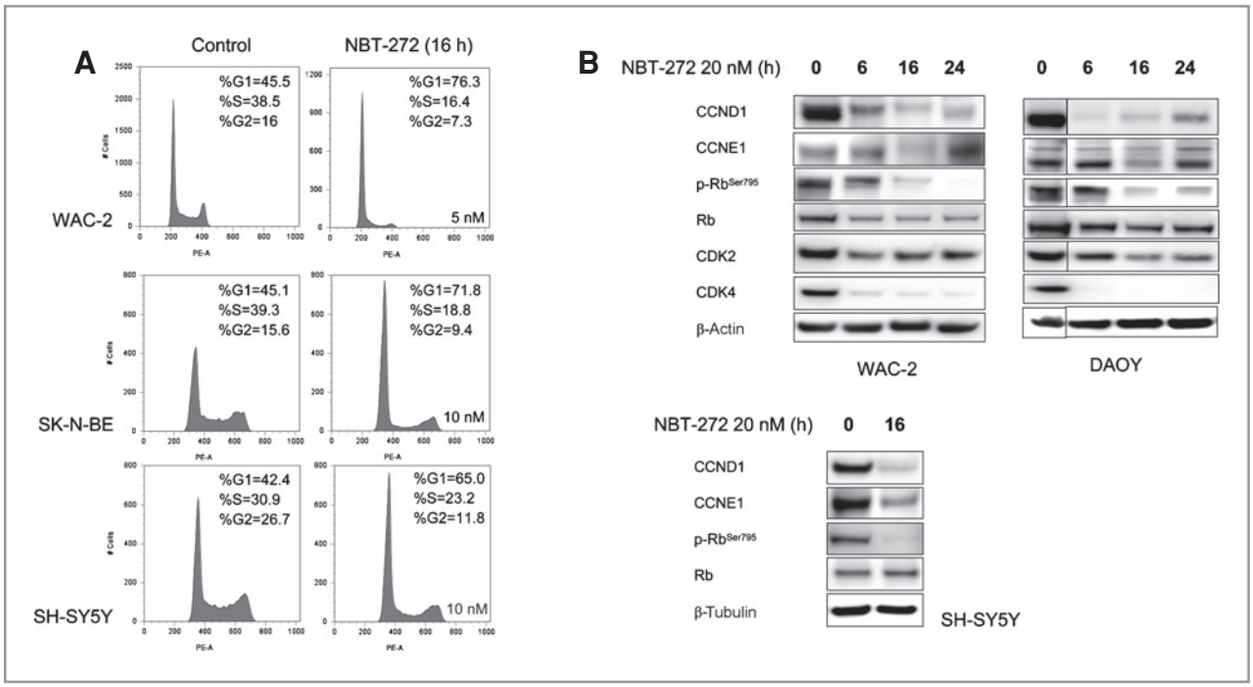

Figure 2. Induction of cell cycle arrest in MB and NB cells. A, WAC-2, SK-N-BE, and SH-SY5Y cells were treated with the indicated amount of NBT-272 for 16 hours or were left untreated (control). The percentage of cells in different stages of the cell cycle is reported in each diagram. B, WAC-2 and DAOY cells were subjected to treatment with 20 nmol/L NBT-272 and analyzed for expression of cell cycle–regulatory proteins by immunoblotting at different time points. The experiment was also confirmed in SH-SY5Y cells treated for 16 hours.

NBT-272-induced cell cycle arrest at the G_1/S transition

To identify which molecular characteristics were responsible for NBT-272's toxicity, we sought to investigate the diverse biological responses triggered in ET cell lines by the compound. The effect on cellular distribution at different stages of the cell cycle was evaluated by DNA staining in MB and NB cells. In dose–response experiments at different time points, 5 nmol/L NBT-272 was already sufficient to almost halve the population of replicating DAOY cells after 24 hours, with a concomitant increase in G_1 population (Supplementary Fig. S3). This observation was found to extend to NB cell lines (WAC-2, SK-N-BE, and SH-SY5Y; Fig. 2A). Here, NBT-272 induced a similar effect after 16-hour treatment, thus suggesting that blocking the cell cycle progression at the G_1 to S transition occurs as one of the earliest mechanisms leading to proliferative arrest.

Consistent with the data obtained by cytofluorimetry, NBT-272 induced loss of CCND1 and of its interacting partner the cyclin-dependent kinase (CDK)4 (Fig. 2B). These proteins are both important regulators of the cell cycle progression through the G_1 phase (26), along with the phosphorylation status of the retinoblastoma protein, which changes throughout the cell cycle with highest levels when cells enter the S phase (27). Consistent with an arrest in the G_1/S transition, RB was hypophosphorylated on treatment with NBT-272 (Fig. 2B). On the contrary, the effect on CCNE1/CDK2 was transient.

Two NB cell lines (SH-SY5Y and SK-N-BE) and the MB cells DAOY and D341 were treated for 24 hours with 20 nmol/L NBT-272, followed by expression profiling of 84 cell cycle–related genes by qRT-PCR. Supplementary Fig. S4A illustrates the statistically significant gene expression changes induced by NBT-272. The 23 transcripts fell into 3 functional categories: (1) G_1/S transition of the cell cycle; (2) DNA damage (e.g., growth arrest and DNA damage-inducible alpha protein, GADD45A); and (3) regulation of transcription elongation. The most relevant changes concerned genes involved in the G_1 arrest, in support of the data from our biological assays (Fig. 2). For instance, consistent with a G_1/S block of the cell cycle, the mRNA level of CDK2 decreased, whereas the inhibitor molecules CDKN1A/$p21^{Cip1}$ and CDKN1B/$p27^{Kip}$ were induced on treatment (Supplementary Fig. S4A). Whereas CCND1 and CCNE1 were regulated solely at the protein level (Fig. 2B), other cyclins and CDK molecules involved in the RNA polymerase II–mediated gene transcription (CCNC/CDK8, CCNH/CDK7, and CCNT/CDK9) were found to be upregulated.

Apoptosis was induced by NBT-272 in a cell type–dependent manner

Induction of apoptosis was assessed by quantifying the activation of caspase-3/7 induced by NBT-272 (Fig. 3A).

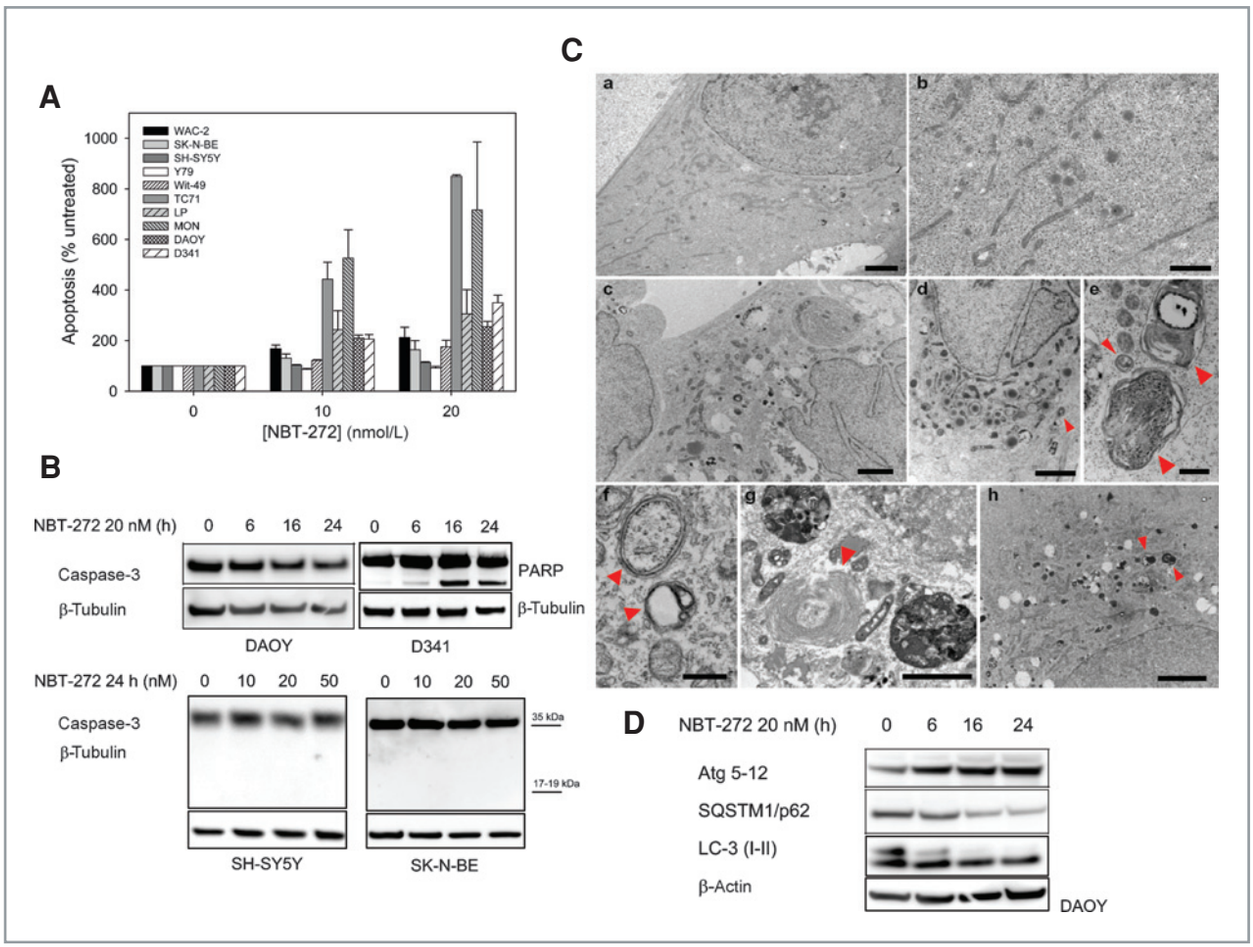

Figure 3. NBT-272-induced apoptosis and autophagy in ET cell lines. A, induction of apoptosis was quantified by Caspase-Glo 3/7 assay (Promega) in 10 cell lines representative of different ET entities following treatment with 10/20 nmol/L NBT-272 for 24 hours. Luminescence signals were normalized to cell viability (MTS assay). B, cleavage of PARP/caspase-3 was evaluated by Western blotting in the MB cell lines D341 and DAOY and in 2 NB cell lines (SH-SY5Y and SK-N-BE), treated as indicated. The apparent molecular weight of full length and cleaved caspase-3 on the blots is indicated (35 kDa, 17–19 kDa). C, high-resolution electron microscopy was done to analyze the effect of NBT-272 (20 nmol/L, 16 hours) on the ultrastructural organization of DAOY cells (a, c–e, and h) and of SK-N-BE cells (b, f, and g). Mock-treated cells (a, b) appeared healthy with homogeneous cytoplasm and regular nuclear envelope. Treated cells displayed a looser cytosol and membrane blebbing at the cell surface (c) and a drastic increase in the amount of vacuoles and lysosomes (c, d). Numerous autophagosomes containing cellular fragments, ribosomes, and whole mitochondria could be detected (arrow heads). DAOY cells were treated separately with rapamycin (200 nmol/L, 48 hours) as control (h). Bars, a, h = 4 μm; b–d, g = 2 μm; and e, f = 0.5 μm. D. Change in expression of the autophagy-related proteins SQSTM1 (p62) and LC-3 and of the Atg 5–12 complex (markers of autophagosome formation) was documented by immunoblotting in DAOY cells.

The proapoptotic response to NBT-272 varied significantly in a cell type– and tumor-dependent way. This observation was also confirmed by Cell Death ELISA assays (Roche; data not shown). Whereas NBT-272 concentrations as small as 10 nmol/L triggered apoptosis in MB-, ESFT-, and MRT-derived cell lines after 24 hours, the same doses had a very modest proapoptotic effect in other cellular models, particularly in NB (Fig. 3A). The limited proapoptotic effect of NBT-272 in MB and NB cells was also shown by immunoblotting (Fig. 3B), where caspase-3 could be only detected as full length protein and PARP cleavage was only induced in MB cell lines (e.g., in D341; Fig. 3B). This observation was confirmed by TUNEL assay, where NBT-272 was able to induce a breakage of the genomic DNA in DAOY cells (Supplementary Fig. S5).

Using a similar approach to that employed for cell cycle–related genes, we investigated the transcriptional changes related to apoptosis triggered by NBT-272 (Supplementary Fig. S4B). Among the differentially expressed genes, we found key components of both the extrinsic and the intrinsic pathways of apoptosis, mainly upregulated on treatment with the compound. This was the case with proapoptotic members of the Bcl-2 family (BIK, BAD, and BAK) and with proteins bearing the caspase recruitment domain (CARD), such as Bcl-10, PYCARD and RIPK2, implicated in the recruitment and binding of caspases. Another major group, namely

genes encoding for ligands and receptors of the tumor necrosis factor (TNF) superfamily, was induced (Supplementary Fig. S4B). It included TNF-α, TNF-β/LTA, TNFSF10/TRAIL, and their corresponding receptors TNFRSF10A and TNFRSF10B. Also the expression of TNFRSF5, TNFRSF9, and TNFRSF6/FAS increased on NBT-272 treatment, altogether indicating enhanced cell responsiveness to death receptor–mediated signaling.

NBT-272 triggered autophagosome formation

Because NBT-272 induced apoptosis with differing efficiency in the ET cell lines tested, we sought additional mechanisms that could lead to cell death. Formation of the distinctive traits of autophagy was investigated at the ultrastructure cellular level by TEM, thus revealing induction of a high number of double-membrane structures containing cellular material (i.e., cytosolic cargo molecules and organelles), described as autophagosomes (28).

In Fig. 3C, TEM images of DAOY (MB) and SK-N-BE (NB) cells are reproduced. Compared with mock-treated controls (Fig. 3C, a and b), treated cells (20 nmol/L NBT-272, 16 hours) displayed all the characteristic signs of autophagy induction, in particular, formation of large autophagosomes (arrow heads in Fig. 3C, e, f, and g) and increased lysosomes and vacuoles. Moreover, the cytosol appeared looser than in control cells, the nuclear envelope became irregular, the mitochondria acquired swollen crests indicative of dysfunctional activity, and myelin figures could be visualized as electron-dense structures. As positive control, DAOY cells were treated separately with rapamycin (200 nmol/L, 48 hours; Fig. 3C, h), inducing autophagy as an event associated with inhibition of mTOR (29). On comparing the effect of the 2 compounds in DAOY cells, NBT-272 proved to be a much stronger inducer of autophagy.

Formation of autophagosomes requires a rather complex rearrangement of Atg proteins, including the protein conjugation system generating the complex Atg5-Atg12 (30), which was detected by immunoblotting, on treatment of DAOY cells with 20 nmol/L NBT-272 (Fig. 3D). Concomitantly, we were able to show the degradation of sequestosome 1 (SQSTM1/p62), that is, a multifunctional adaptor molecule driving the degradation of polyubiquitinated proteins such as those tagged for autophagic clearance (31). Further, we documented the conversion of the microtubule-associated protein light chain 3 (LC3-I) into its membrane-interacting counterpart (LC3-II), which is a well-documented marker of autophagy induction.

MYC depletion resulted as an indirect effect of NBT-272

Because NBT-272 was originally described as a MYC inhibitor, we evaluated the correlation of the expression of c-MYC and MYCN in the ET cell lines (Supplementary Fig. S1) with cell sensitivity to the compound. However, only the MB cell line D341 (bearing *c-MYC* amplification) was significantly more sensitive to NBT-272 than DAOY cells expressing *c-MYC* as single copy gene (Fig. 1; ref. 16), whereas cells from other ET entities displayed a sensitivity similar to NBT-272 (Fig. 1).

Moreover, again regardless of the MYC isoform expressed and the gene copy number of *c-MYC*/*MYCN*, NBT-272 induced depletion of MYC in several ET cell lines, although exclusively at the protein level (Fig. 4A). In contrast, mRNA expression was either left almost unchanged (e.g., *MYCN* in SK-N-BE cells) or was enhanced (e.g., *c-MYC* in D341 cells) by NBT-272 in a dose-dependent manner (Fig. 4A).

The MYC family of transcription factors is known to have short half-lives (32). Therefore, we researched whether NBT-272 could have an effect on protein stability, thus helping to explain MYC depletion. In fact, by using the proteasome inhibitor MG132 (33) in combination with NBT-272, c-MYC was already almost completely rescued after 4 hours (Fig. 4B).

Additional lines of evidence supported an indirect effect of NBT-272 on MYC. Two small-molecule inhibitors specifically targeting the MYC/Max interaction (34, 35) did not show the same profile of activity as NBT-272 (Fig. 4C). In fact, significant cell viability could still be measured at high doses of both Mycro3 and 10058-F4. In particular, WAC-2 cells were almost unaffected by either of the compounds, whereas 10^3 times less concentrated NBT-272 completely arrested cellular growth.

Finally, only a limited number of gene expression changes induced by NBT-272 matched with the transcriptional effects of siRNA-mediated silencing of *c-MYC* in DAOY cells (Supplementary Fig. S6; Fig. 4D). In fact, only a restricted number of genes were up-/downregulated in both sets of experiments, that is, 11 apoptosis-related and 11 cell cycle–related transcripts (out of 84 in each analysis). Among them, even fewer genes were known direct targets of c-MYC (i.e., GADD45A, BIRC3, DFFA, CDK6, CDKN1A/p21, and PCNA). On the other hand, genes reported to be MYC targets exhibited a different expression profile, that is, they were mostly downregulated in *c-MYC*–depleted cells, but induced or left unchanged in cells treated with NBT-272 (e.g., the cyclins CCNB1, CCNB2, CCND2, CCNH, and the DNA replication regulators MCM4 and MCM5).

Altogether, these results led us to conclude that a direct inhibition of MYC is unlikely to explain the effects triggered by NBT-272 in ET cells, which are more likely to be the result of more complex mechanisms and of interference with upstream pathways.

NBT-272 targeted components of the AKT and ERK signaling pathways

To improve our understanding of the regulatory mechanisms leading to arrest of the cell cycle, apoptosis, and autophagy, we analyzed the activation status of 2 signaling pathways, that is, the AKT/mTOR and the MEK/ERK pathways, regulating these cellular responses

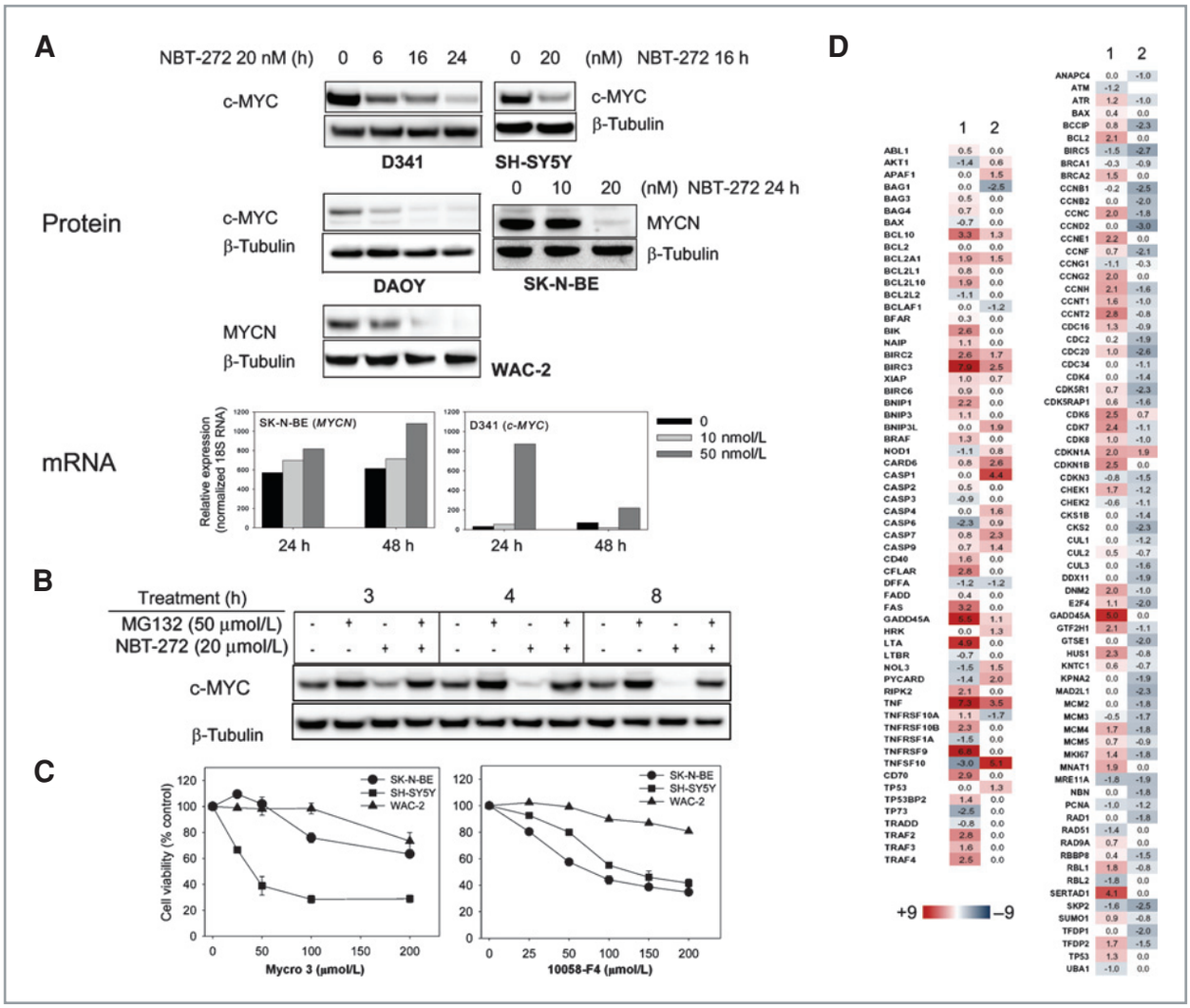

Figure 4. Indirect effect of NBT-272 on MYC. A, the effect of NBT-272 on either c-MYC or MYCN in different ET cell lines was analyzed at the protein (Western blotting) and at the mRNA level (qRT-PCR). B, cotreatment of D341 cells with MG132, inhibiting proteasome-mediated protein degradation, rescued c-MYC from the depleting effect of NBT-272. C, cell viability was evaluated in 3 NB-derived cell lines (SK-N-BE, SH-SY5Y, WAC-2) incubated for 72 hours with increasing doses of Mycro3 (34) or 10058-F4 (35). D, changes in the expression of apoptosis- (left) and cell cycle–related genes (right) were analyzed comparing the effect of NBT-272 (1) and of *c-MYC* downregulation by siRNA (2). For the silencing experiment, DAOY M2 cells were transfected transiently with *c-MYC* siRNA and gene expression was analyzed after 48 hours by using the RT^2 Profiler PCR Array. The same cell line was treated for 24 hours with 20 nmol/L NBT-272 and subjected to the same analysis. Results in the hit maps are expressed as the $\log_2$ ratio (where ratio is $2^{-\Delta\Delta Ct}$) obtained from NBT-272-treated or *c-MYC* siRNA-transfected cells normalized to the corresponding controls (i.e., mock-treated and control siRNA-transfected cells, respectively). Values in each analysis represent the average of 3 independent experiments.

(36). Furthermore, both pathways have been documented to be relevant also in the development of ET.

Figure 5A shows the effect of NBT-272 on the activation status of AKT varying in different cell lines, with phosphorylation at Ser473 significantly decreasing in WAC-2, SK-N-BE, and SH-SY5Y cell lines, but slightly enhanced in DAOY cells. On the other hand, the level of phosphorylated ERK 1/2 (p44/p42) diminished in all cell lines tested, although with different kinetics (Fig. 5A), suggesting a different sensitivity of the 2 pathways to NBT-272. To test whether NBT-272 had a direct effect on the enzymatic activity of either AKT or ERK and could thus be regarded as a kinase inhibitor, we quantified the *in vitro* kinase activity of 271 purified recombinant kinases (Supplementary Fig. S7A). However, none of them were significantly impaired by NBT-272 (20 nmol/L). Additionally, different subunits and isoforms of phosphoinositide-3 kinase (PI3K) were tested (Supplementary Fig. S7B), again showing no effect from NBT-272 (20 nmol/L).

Although the exact mechanism of action of NBT-272 remained unsolved, the compound clearly affected AKT- and ERK-dependent cellular functions. The downstream target of ERK, MNK1 (37), was also deactivated on treatment with NBT-272, whereas the upstream kinase

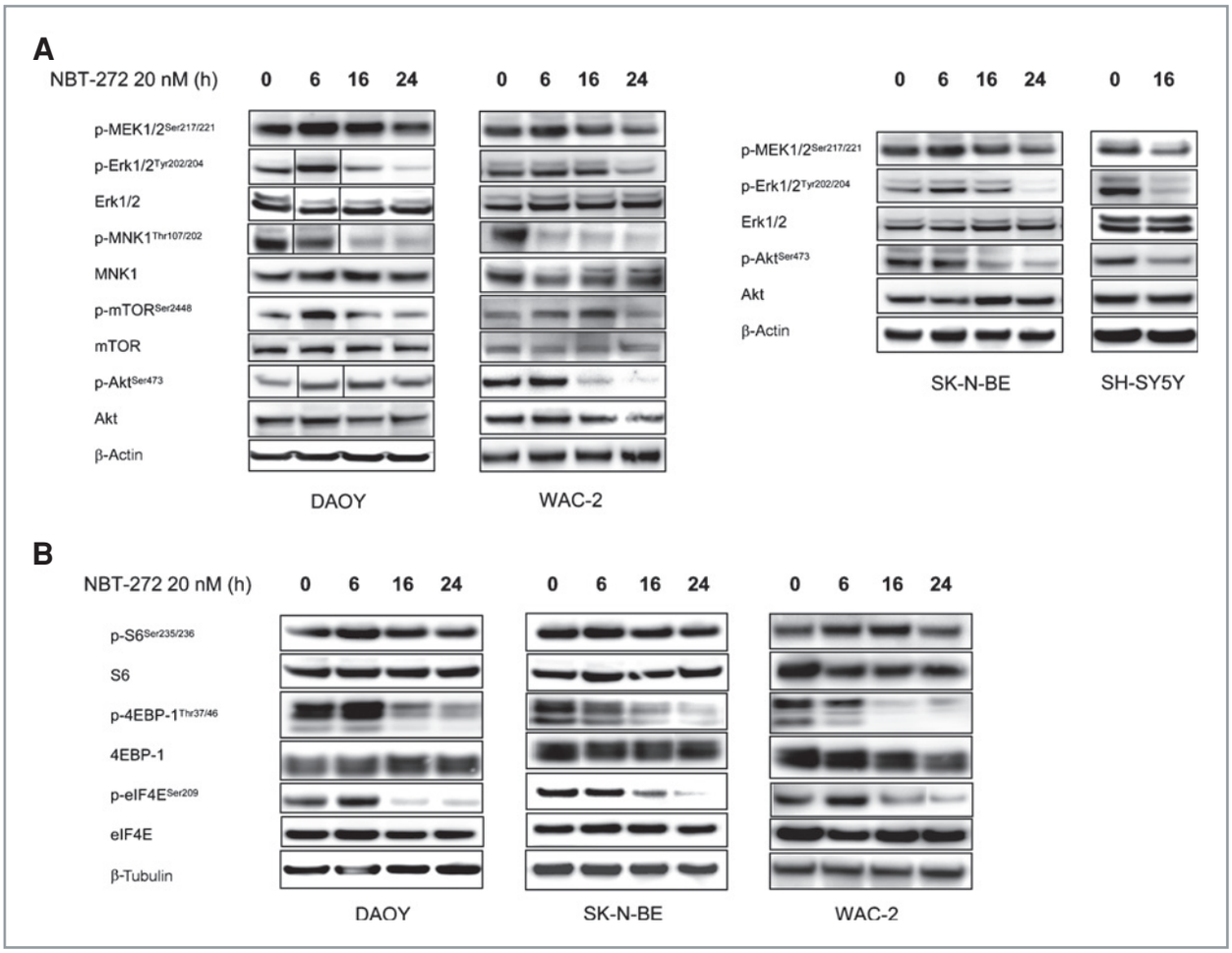

Figure 5. Inhibition of protein translation and of the AKT/mTOR- and ERK-dependent pathways. A, protein expression and activation status of components of the AKT/mTOR and ERK pathways were investigated by immunoblotting. B, treatment with NBT-272 triggered a depletion of active eIF4E and 4EBP-1 proteins in different cell lines as early as 16 hours.

MEK1/2 was only slightly affected (Fig. 5A). MNK1 is known to phosphorylate both eIF4E and its repressor, 4EBP-1 (38). 4EBP-1 is also a direct target of mTOR, playing a key role as regulator of protein translation downstream of AKT (22). Interestingly, regardless of the AKT activation status, phosphorylation of both eIF4E and 4EBP-1 was impaired by NBT-272 in MB and NB cells (Fig. 5B), thus preventing the cap-dependent protein translation machinery from being completely functional. Additionally, the phosphorylation status of the ribosomal S6 protein, target of mTOR via S6 kinase (S6K; ref. 39), was proven to be unaffected, thus reinforcing the idea of a 4EBP-1–dependent block of protein synthesis.

Moreover, we asked whether the cellular sensitivity to NBT-272-mediated G_1/S block of the cell cycle, apoptosis, or autophagy would vary if 1 of the pathways had been affected by using specific inhibitors. To test the relevance of the mTOR/AKT pathway, BEZ235 was used (40). A pretreatment with the PI3K/mTOR inhibitor increased both depletion of CCND1 and degradation of SQSTM1/p62 when compared with NBT-272 alone (Supplementary Fig. S8A), whereas no significant enhancement of the proapoptotic functions of NBT-272 was observed (Supplementary Fig. S8B). Although BEZ235 alone had apparently no effect, under these conditions, on the expression of the cell cycle and autophagy markers in DAOY cells, the inhibitor was functional, as shown by the strong deactivation of AKT (Supplementary Fig. S8A) and the induction of apoptosis (Supplementary Fig. S8B).

NBT-272 blocked tumor progression in a xenograft model of NB

NB cells bearing a transgene encoding luciferase (SH-SY5Y-LUC) were injected in the flank of 10 athymic nude mice and were allowed to develop for 3 weeks. The effect of the intraperitoneal administration of NBT-272 (10 μmol/L per week) and of the vehicle (0.003% EtOH/PBS) on the tumor growth was monitored by quantifying the luminescent signal from engrafted SH-SY5Y-LUC cells. After 6 weeks of treatment, the tumors almost

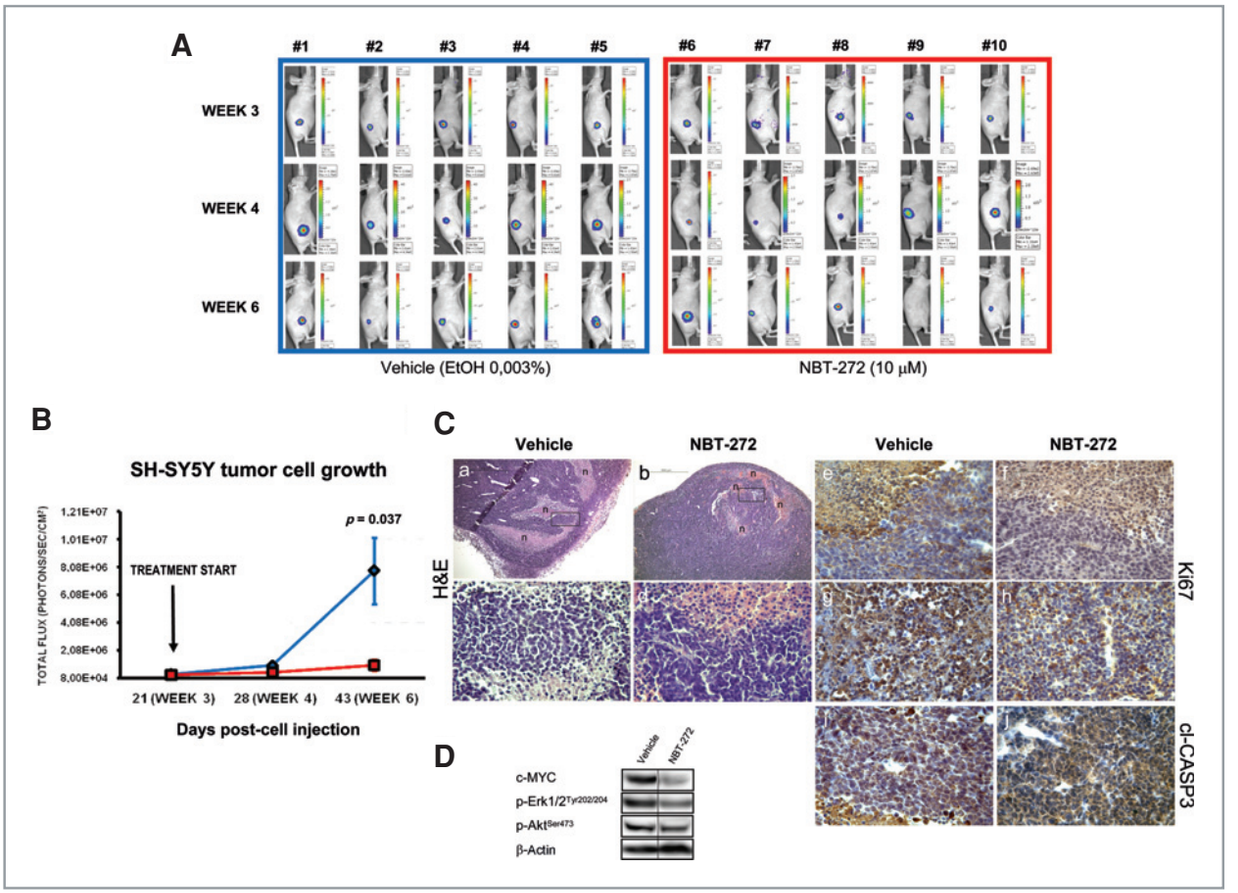

Figure 6. Reduction of tumor growth induced by NBT-272 in a xenograft model. Female athymic nude mice were injected in the right flank with SH-SY5Y-LUC cells. Three weeks after injection, the mice were divided into 2 homogeneous groups (based on the amount of engrafted human cells). The mice received either NBT-272 (10 μmol/L per week) or vehicle (0.003% EtOH/PBS), both administered 3 times a week. A, the tumor growth rate was monitored on a weekly basis by quantifying the luciferase activity of the recombinant tumor cells *in vivo* (see scale bars) and plotted starting from the first day of treatment. Blue, vehicle-treated animals. Red, NBT-272-treated animals. BLI data are available as a supplementary figure (see Supplementary Fig. 7S). B, quantification of the tumor growth rate. C, H&E and immunohistochemistry staining of tumor samples obtained from mice treated either with vehicle alone (left panels) or with NBT-272 (right panels) according to the described protocol. The panels c and d represent the enlarged view of the selected areas in panels a and b, respectively. In a and b, the tumors are shown as hyperdense-rounded structures with some necrosis portions (n). In panels e–h, the tumor sections were stained for Ki67 (proliferation), whereas in panels i and j the staining for cleaved caspase-3 (cl-CASP3, apoptosis) is shown. Images a and b, ×5 magnification; images c–j, ×40 magnification. D, Western blotting detection of c-MYC, p-ERK1/2, and p-AKT was done in total protein extracts from tumors developed by mice 4 (vehicle treated) and 9 (NBT-272 treated).

stopped growing in the mice receiving NBT-272, whereas the human NB SH-SY5Y cells expanded into large tumors in the control animals (Fig. 6A and B; Supplementary Fig. S9). In fact, a 6.3-fold increase of tumor size could be detected in the control group compared with the NBT-272–treated group ($P = 0.037$ according to Student's t test). The result clearly indicated that NBT-272 has a significant antiproliferative effect on the tumor development *in vivo*. The hemotoxylin/eosin staining of some representative tumor samples showed a compact and rounded shape of the tumors, which were characterized by a very dense cell distribution with some necrotic areas (Fig. 6C, a–d). Tumors from NBT-272-treated mice showed a clear reduction of Ki67-positive cells (Fig. 6C, f and h), whereas a prominent cellular perinuclear staining in vehicle-treated mice was observed (Fig. 6C, e and g), thus confirming the significant antiproliferative effect of NBT-272 also *in vivo*. Activation of caspase-3 was also evaluated (Fig. 6C, i and j), which shows that the treatment with NBT-272 did not induce a significantly higher induction of apoptosis than with control tumors. This result confirmed our findings *in vitro* (Fig. 3A and B) by referring to the same cellular model, that is, SH-SY5Y cells, which were used to induce the tumors. Finally, NBT-272 treatment had an effect on MYC stability/expression in the tumors, in which ERK activation was also impaired (Fig. 6D).

Discussion

A fine regulation of protein turnover by a tunable modulation of protein stability requires specific post-

translation modifications (such as phosphorylation) of key molecules. Many of these molecules are significant components of prosurvival pathways and often play a crucial role in tumor development and progression, in conditions of constitutive and growth factor–independent activation. In past decades, great efforts have been made to interfere with those signaling pathways by employing different strategies of targeted therapy, ranging from antibodies against receptor tyrosine kinases (RTK) to small-molecule inhibitors blocking downstream signaling effectors. Downstream of RTK activation, the induction of MYC transcription factors and of their pleiotropic effects on cell growth has been frequently associated with neoplastic transformation. Therefore, MYC itself clearly represents an attractive target for anticancer therapy (41), also due of its correlation with poor prognosis and high-grade malignancy in many types of tumors, including pediatric malignancies (10, 11, 42, 43).

In the context of MYC-overexpressing ET tumors, we wanted to investigate the biological responses of a group of ET-derived cell lines to NBT-272, a small molecule of about 550 Da, previously described as inhibitor of MYC (15, 16). Its toxicity and ability to prevent colony formation *in vitro* indicated a very strong effect of NBT-272 at low concentrations.

In the search for an association between toxicity of NBT-272 and MYC expression levels in different ET cell lines, no significant correlation could be found. This observation led us to question any direct effect of NBT-272 on the oncogene, which would more likely be the result of different mechanisms, such as interference with protein synthesis and stability, affecting also the MYC family members known to have short half-lives (32). Indeed, we could observe that inhibition of the proteasome-dependent protein degradation pathway restored the depleting effect of NBT-272 and almost completely stabilized c-MYC. Moreover, the effect induced by 2 specific MYC/Max inhibitors (34, 35) and by *c-MYC* silencing in ET cells did not match the cellular response to NBT-272, again detrimental to the hypothesis of a direct effect on MYC. This conclusion, however, certainly does not exclude that MYC inhibition contributed to the panel of cellular responses observed on NBT-272 treatment.

Even though the compound is not a direct kinase inhibitor, our study indicated the ability of NBT-272 to interfere with the activation status of different components of the AKT/mTOR and MEK/ERK signaling pathways. We therefore attributed the effect of NBT-272 on apoptosis, cell cycle, and autophagy to its ability to interfere with these pathways. Indeed, the basal level phosphorylation of ERK1/2, MNK1, and AKT in MB and NB cells was impaired by NBT-272, under the same conditions that induced efficient cell cycle arrest (G_1/S) and autophagosome formation. It is also relevant to emphasize that the 2 pathways converge to regulate the activation status of eIF4E and 4EBP-1, both dephosphorylated (i.e., inactive) in the presence of NBT-272, thus leading us to speculate that targeting these pathways would prevent full activation of the cap-dependent protein translation machinery. Furthermore, MYC depletion could possibly be explained, not only as an effect of protein synthesis inhibition, but also as a consequence of decreased phosphorylation at its Ser62 residue, which has a stabilizing effect and is catalyzed by ERK (44).

In total, our observations strongly suggest that both the AKT and MEK/ERK signaling pathways participate in orchestrating the cellular responses to NBT-272, at least *in vitro*. Moreover, combination of NBT-272 with a specific inhibitor of PI3K/mTOR, BEZ235 (40), seemed to further increase the NBT-272–dependent effect on cell cycle and autophagy, but not apoptosis. Numerous components of the AKT/mTOR and MEK/ERK pathways have been found deregulated in cancer, including ET. High basal activation levels of AKT and of the S6 ribosomal protein were documented in MB (45, 46) and a relevant role in NB pathogenesis was attributed to PI3K, found to be overexpressed in NB tumors (47). Phosphorylation of AKT, S6, and ERK has also been reported as a frequent event in NB, in which AKT activation in particular was correlated with diverse indicators of aggressive disease and poor prognosis (48). These 2 signaling pathways were also negatively associated with malignancy in childhood rhabdomyosarcoma (49) and in Ewing's sarcoma (50), thus rendering it certainly worthwhile to promote the idea of developing common therapeutic strategies effective in different ET entities.

Finally, NBT-272 arrested tumor growth in a xenograft model of human NB, showing that the potent effect of this compound could also be reproduced *in vivo*, with concomitant reduction of MYC expression and of ERK activation in the treated tumors. These very encouraging results warrant the extension of investigations to more ET models.

In summary, NBT-272 induced a relatively complex pattern of cellular responses, including inhibition of crucial regulators of protein synthesis. All these events could be related functionally to interference with key cell survival pathways (AKT and MEK/ERK) playing a role in the pathogenesis of several ETs. Our findings increase the present level of understanding of the mechanisms of action of NBT-272, explaining its potent antitumor effect in *in vitro* and *in vivo* models of ET. This study certainly justifies further efforts to define more clearly the potential benefits of using NBT-272 in novel therapeutic strategies for pediatric tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Research Article

Molecular Cancer Therapeutics

Disabling *c-Myc* in Childhood Medulloblastoma and Atypical Teratoid/Rhabdoid Tumor Cells by the Potent G-Quadruplex Interactive Agent S2T1-6OTD

Tarek Shalaby[1], André O. von Bueren[1], Marie-Louise Hürlimann[1], Giulio Fiaschetti[1], Deborah Castelletti[1], Tera Masayuki[3], Kazuo Nagasawa[3], Alexandre Arcaro[1], Ilian Jelesarov[2], Kazuo Shin-ya[4], and Michael Grotzer[1]

Abstract

We investigated here the effects of S2T1-6OTD, a novel telomestatin derivative that is synthesized to target G-quadruplex–forming DNA sequences, on a representative panel of human medulloblastoma (MB) and atypical teratoid/rhabdoid (AT/RT) childhood brain cancer cell lines. S2T1-6OTD proved to be a potent *c-Myc* inhibitor through its high-affinity physical interaction with the G-quadruplex structure in the *c-Myc* promoter. Treatment with S2T1-6OTD reduced the mRNA and protein expressions of *c-Myc* and *hTERT*, which is transcriptionally regulated by *c-Myc*, and decreased the activities of both genes. In remarkable contrast to control cells, short-term (72-hour) treatment with S2T1-6OTD resulted in a dose- and time-dependent antiproliferative effect in all MB and AT/RT brain tumor cell lines tested (IC_{50}, 0.25–0.39 μmol/L). Under conditions where inhibition of both proliferation and *c-Myc* activity was observed, S2T1-6OTD treatment decreased the protein expression of the cell cycle activator cyclin-dependent kinase 2 and induced cell cycle arrest. Long-term treatment (5 weeks) with nontoxic concentrations of S2T1-6OTD resulted in a time-dependent (mainly *c-Myc*–dependent) telomere shortening. This was accompanied by cell growth arrest starting on day 28 followed by cell senescence and induction of apoptosis on day 35 in all of the five cell lines investigated. On *in vivo* animal testing, S2T1-6OTD may well represent a novel therapeutic strategy for childhood brain tumors. *Mol Cancer Ther; 9(1); 167–79. ©2010 AACR.*

Introduction

Medulloblastomas (MB) are the most common malignant pediatric neoplasms of the central nervous system (CNS) and represent >20% of all pediatric brain tumors (1). With current treatment strategies, nearly half of all patients will eventually die from progressive tumors. CNS atypical teratoid/rhabdoid tumors (AT/RT) are rare but highly malignant embryonal tumors in young children (2–5). Experience to date indicates that infants and children with CNS AT/RT respond very poorly to chemotherapy and radiotherapy (6–9). Accordingly, the identification of novel therapeutic strategies for MB and AT/RT remains a major goal.

Authors' Affiliations: [1]Department of Oncology, University Children's Hospital Zurich; [2]Department of Biochemistry, University of Zurich, Zurich, Switzerland and [3]Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology; [4]National Institute of Advanced Industrial Science and Technology, Tokyo, Japan

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Corresponding Author: Michael Grotzer, University Children's Hospital Zurich, Steinwiesstrasse 75, 8032 Zurich, Switzerland. Phone: 41-44-266-71-11; Fax: 41-44-266-71-71. E-mail: Michael.Grotzer@kispi.uzh.ch

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Telomestatin is a natural G-quadruplex–intercalating agent isolated from *Streptomyces anulatus* 3533-SV4 (10). Telomestatin is a potent telomere maintenance-disabling drug in cervical carcinoma, breast cancer, multiple myeloma, leukemia, and neuroblastoma cells (11–14). Telomestatin suppresses cellular proliferation and induces apoptosis within a few days in various cancer cells but not in normal cells (15). Telomestatin has a characteristic macrocyclic ring system that consists of sequential pentaoxazoles, bismethyloxazoles, and a thiazoline. It has been suggested that these structural features of telomestatin strongly bind to G-quartets by overlapping, and this interaction is believed to promote the formation and stabilization of G-quadruplex structures (16). To increase this interaction, installation of additional functional groups, such as carbonyl, hydroxyl, and/or amino groups, would be required. Based on this concept, telomestatin derivatives were designed, and S2T1-6OTD was synthesized as a derivative bearing a macrocyclic bisamide (15). Based on its three-dimensional structure, it had been speculated that S2T1-6OTD has a high affinity for G-quadruplex–forming sequences, including the nuclease hypersensitivity element III1 (17). This is a major transcriptional control element in the *c-Myc* promoter region, and it has been found to control up to 85% of total *c-Myc* transcription (17–23).

c-Myc is a pleiotropic transcription factor that has been linked to diverse cellular functions, such as cell cycle

regulation (24), proliferation (25), and growth (26), and it has an important regulatory effect on telomerase activity (27, 28) that promotes immortalization. Aberrant *c-Myc* signaling has been observed in human cancers, and *c-Myc* has been shown to promote cell transformation and tumor progression (29–33). In childhood MB, high *c-Myc* mRNA expression and *c-Myc* gene amplification have been suggested as indicators of poor prognosis (33–43). Furthermore, high *c-Myc* mRNA expression was shown to be significantly associated with tumor anaplasia (44, 45).

In this study, we examined the short- and long-term effects of S2T1-6OTD in a representative set of childhood MB and AT/RT cells.

Materials and Methods

Tumor and Control Cells

DAOY human MB cells, CA46 and Ramos human Burkitt's lymphoma cells, PC12 rat pheochromocytoma cells, and MRC-5 human untransformed fibroblast cells were purchased from the American Type Culture Collection. D341 and D425 MB cells were the kind gift of Dr. Henry Friedman (Duke University, Durham, NC). DAOY M2 (c-Myc vector–transfected) human MB cells have been described previously (45). BT-12 and BT-16 human CNS AT/RT cells were the kind gift of Dr. Peter Phillips (The Children's Hospital of Philadelphia, Philadelphia, PA). Rat fibroblast cells HO15.19 [*c-Myc* knockout (−/−)] and TGR-1 *c-Myc* wild-type (rat fibroblast) were the kind gift of Prof. B. Amati (European Institute of Oncology, Milan, Italy). DAOY, D341, and D425 cells were cultured in Richter's zinc option medium/10% fetal bovine serum (FBS; 1% nonessential amino acids were added to the medium for D341 and D425 cells and G418 was added to the medium for DAOY M2 to a concentration of 500 μg/mL). BT-12 and BT-16 cells were cultured in DMEM (with Glutamax)/10% FBS. CA46 and Ramos cells were grown in RPMI 1640 supplemented with 10% FBS. MRC-5 cells were grown in MEM supplemented with 5% FBS. PC12 cells were cultured in complete growth medium with FBS to a final concentration of 2.5% horse serum. HO15.19 and TGR-1 cells were cultured in DMEM with 8% calf serum. BJ foreskin fibroblasts were cultured in DMEM containing 10% iron-supplemented calf serum. All cell cultures were maintained at 37°C in a humidified atmosphere with 5% CO_2. Cell lines that were not purchased from the American Type Culture Collection in 2009 were tested for their authentication by karyotypic analysis using molecular cytogenetic techniques, such as comparative genomic hybridization.

PCR-Stop Assay

PCR-stop assay was done as previously reported (46). Oligonucleotides ss-telo24 d[TTAGGGTTAGGGTTAGGGTTAGGG], ss-Pu22 d[GAGGGTGGGGAGGGTGGGGAAG], mutated mut-ss-Pu22 d[GAGGGT*AA*GGAGGGTGGGGAAG], and the complementary sequences of telo24 d[TCTCGTCTTCCCTAA] (telo24 rev) and ss-Pu22 d[ATCGCTTCTCGTCTTCCCCA] (ss-Pu22 rev) were used. The chain extension reaction was done in 1× PCR buffer containing 0.2 mmol/L deoxynucleotide triphosphate, 5 units Taq polymerase, 7.5 pmol oligonucleotides, and various concentrations of S2T1-6OTD and telomestatin. The mixtures were incubated in a thermocycler under the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 20 s, 47°C for 20 s, and 72°C for 20 s. Amplified PCR products were resolved on 12% native polyacrylamide gels in 0.5× Tris-borate EDTA buffer and stained with ethidium bromide. The IC_{50} values were calculated based on the fluorescence intensity scanned with a phosphorimager (Typhoon 8600, Molecular Dynamics).

Circular Dichroism Spectroscopy

Circular dichroism (CD) experiments were carried out with a Jasco J-715 instrument equipped with a computer-controlled water thermostat. Optical path length was 1 mm. The spectra were calculated with J-715 Standard Analysis software (Japan Spectroscopic Co.). Each spectrum was recorded thrice, and the reported spectra represent the average of three scans at 5 nm/min and data pitch 0.2 nm. ss-Pu22 (GAGGGTGGGGAGGGTGGGGAAG) or ss-Telo24 d$[TTAGGG]_4$ was diluted by Tris buffer (50 mmol/L, pH 7.0) at a concentration of 10 μmol/L. S2T1-6OTD was dissolved with 10 mmol/L stock solution and titrated into the DNA samples at 5 mol equivalent (the 10 mmol/L stock solution of S2T1-6OTD was made up in 10% DMSO–90% water). The DNA strand concentrations were 10 μmol/L, and the CD data are a representation of three averaged scans taken at 25°C. All CD spectra are baseline corrected for signal contributions due to the buffer and for buffer and DMSO for the samples containing S2T1-6OTD. Thermal melting experiments were done using 10 μmol/L of either ss-Pu22–forming or ss-Telo24–forming sequence in the presence of 50 μmol/L S2T1-6OTD in a Tris-HCl buffer (50 mmol/L, pH 7.4), and the ellipticity at 264 nm was monitored on continuous heating at 2°C/min between 20°C and 95°C.

Real-time Quantitative Reverse Transcription-PCR

Isolation of total RNA, cDNA synthesis, and kinetic real-time PCR quantification of *hTERT* and *c-Myc* mRNA were done as previously described (47, 48). Experiments were done in triplicate for each data point. The amount of hTERT and *c-Myc*, normalized to the endogenous control 18S rRNA, was related to the commercially available calibrator human cerebellum (Clontech).

Western Blot Analysis

S2T1-6OTD–treated and control MB and AT/RT cells were washed twice in medium, centrifuged, and lysed in buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 1% Triton X-100, 1% NP40, 0.1% SDS, 5 mmol/L EDTA] for 20 min on ice followed by centrifugation at 10,000 × *g* for 15 min. The crude lysates were heated for 5 min at

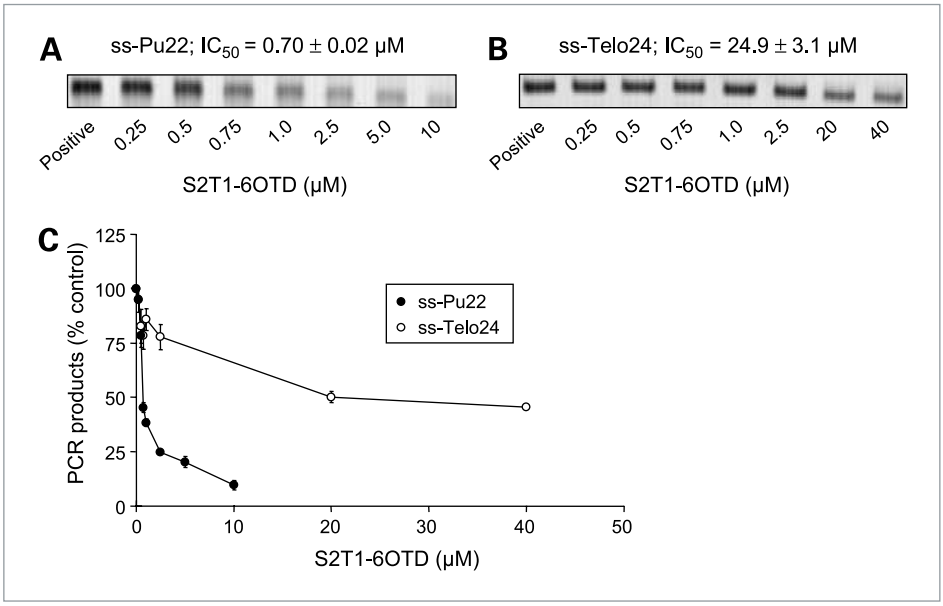

Figure 1. PCR-stop assay products in the presence of increasing concentrations of S2T1-6OTD and either G-quadruplex–forming single-stranded *c-Myc* promoter sequence ss-Pu22 (**A**) or telomeric ssDNA sequence ss-Telo24 oligomer (**B**). **C,** quantification of the band absorbance (fluorescence intensity) by using phosphorimager. *Points*, mean of three independent experiments; *bars*, SD. PCR-inhibitory activities were calculated by the following equation: (intensity of the band in the presence of S2T1-6OTD)/(intensity of positive control band).

95°C in the presence of 3% mercaptoethanol. Equal amounts of protein (30 μg/lane) from the homogenates were separated by 10% (w/v) SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Nonspecific binding sites were blocked with 10 mmol/L TBS containing 0.1% Tween 20 and 10% nonfat milk. Membranes were then incubated overnight at 4°C with anti–*c-Myc* monoclonal antibody (Cell Signaling Technology, Inc.), anti–cyclin-dependent kinase 2 (CDK2) monoclonal antibody (Santa Cruz Biotechnology), or anti–β-actin monoclonal antibody (LS2T1-6OTD; Abcam). Membranes were then washed thrice at room temperature, and bound immunoglobulin was detected with anti-isotype monoclonal antibody coupled to horseradish peroxidase (Santa Cruz Biotechnology). The signal was visualized by enhanced chemiluminescence (Amersham Biosciences) and autoradiography. Relative band intensities were determined using Quantity One analysis software (Bio-Rad).

c-Myc Transcription Factor Binding Activation Assay

Nuclear protein extracts were obtained from S2T1-6OTD–treated and S2T1-6OTD–untreated MB and AT/RT cells by using the BD TransFactor Extraction kit (BD Clontech) as described previously (49). The activation of *c-Myc* was measured by using the Mercury TransFactor assay (BD Clontech), an ELISA-based assay (50), as described previously (49).

Cell Cycle Analysis

MB and AT/RT cells were washed with PBS, fixed in 70% ethanol, and kept at −20°C for at least 24 h. They were then washed in PBS and resuspended in 50 mg/mL propidium iodide and RNase (10 mg/mL) in PBS. The cell suspension was incubated for 30 min at room temperature, and cell cycle distribution was determined by flow cytometry (FACSCalibur, Becton Dickinson), with CellQuest software analysis and quantification using ModFit software as described previously (49).

Cell Growth Assays

For long-term cell growth studies, cells were seeded at 1×10^4/mL (5 mL) into a 25-cm^2 tissue culture flask in the presence or absence of subtoxic doses of S2T1-6OTD. Subtoxic dose was defined as the concentration inhibiting a maximum of 10% human MB cells grown on a 72-h drug exposure. Cells were then cultured for 4 d and then trypsinized and counted. At each passage, 1×10^4 cells/mL were replated into a new culture flask with fresh medium containing drug solution. Results were expressed as the cumulated population doublings as a function of the time of culture, as described by Binz et al. (14) and Riou et al. (51). For cytotoxicity assay, cell viability was quantified using a colorimetric MTS assay (Promega) as previously described (47, 52). Each condition was done in triplicate.

Telomerase Activity

Telomerase activity in human MB and AT/RT cell lines was measured by using the TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics) as previously described (14, 47). All samples were assayed in triplicate.

Telomere Length

Telomere length in MB and AT/RT cells was determined by using the TeloTAGGG Telomere Length Assay (Roche Diagnostics) for measuring the length of telomere restriction fragments as previously described (47).

Apoptosis Assay

A photometric enzyme immunoassay (Cell Death Detection ELISA, Roche Diagnostics) was used for the

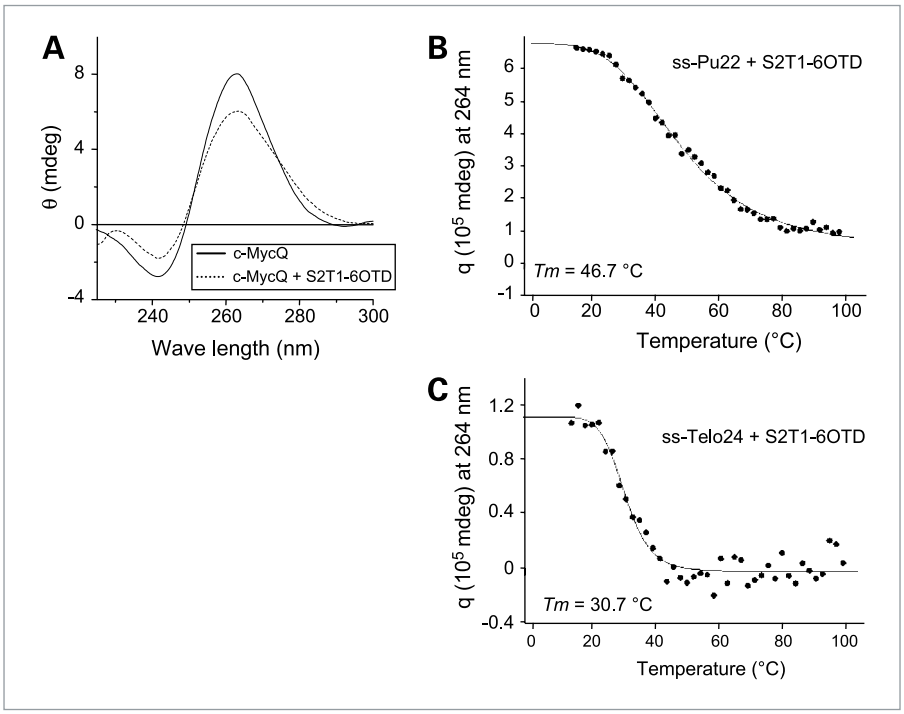

Figure 2. **A,** CD spectra of c-MycQ in the presence or absence of S2T1-6OTD (50 μmol/L). The c-MycQ–forming oligonucleotide concentration was 10 μmol/L. Spectra were recorded in 50 mmol/L Tris (pH 7.2) and 100 mmol/L KCl at 25°C. **B,** CD melting curves of ss-Pu22 (10 μmol/L) at 265 nm in the presence of S2T1-6OTD (50 μmol/L). **C,** CD melting curves of ss-Telo24 (10 μmol/L) at 265 nm in the presence of S2T1-6OTD (50 μmol/L).

quantitative determination of cytoplasmic histone-associated DNA fragments, as described previously (53).

Statistical Analysis

All data are expressed as mean ± SD. Student's *t* test was used to test statistical significance. $P < 0.05$ was considered to be significant. GraphPad Prism 4 (GraphPad Software) software was used to calculate IC_{50} values and their 95% confidence intervals and to statistically compare the fitted midpoints (log IC_{50}) of the two curves.

Results

S2T1-6OTD Structure

Supplementary Fig. S1A shows the chemical structure of S2T1-6OTD compared with the well-characterized telomestatin. The structure of S2T1-6OTD was confirmed by ^{1}H nuclear magnetic resonance (Supplementary Fig. S1B) and high-resolution mass spectrometry (Supplementary Fig. S1C).

Stabilization of *c-Myc* G-Quadruplex Structures by S2T1-6OTD

To test whether S2T1-6OTD is able to stabilize the *c-Myc* G-quadruplex in the guanine-rich sequence of the *c-Myc* promoter, we used PCR-stop assay (54). In the presence of increasing concentrations of S2T1-6OTD, the 5′ to 3′ DNA extension of the ssDNA of the *c-Myc* promoter sequence ss-Pu22 oligomer was inhibited in a dose-dependent manner, indicating the stabilization of the G-quadruplex structure by S2T1-6OTD with a low IC_{50} value of 0.70 ± 0.02 μmol/L (Fig. 1A). No significant inhibition was observed when ss-Pu22 mutant (containing a mutation in two guanine repeats into aniline) was used (data not shown).

Because potential G-quadruplex–forming sequences exist not only in the *c-Myc* promoter region but also in the telomeric ssDNA that proved to be recognized and specifically stabilized by telomestatin, the parental compound of S2T1-6OTD (10), we investigated the selectivity of S2T1-6OTD to stabilize the G-quadruplex in the *c-Myc* promoter ss-Pu22 over the telomeric ssDNA sequence ss-Telo24 by the PCR-stop assay. The results showed that inhibition of ss-Telo24 fragment extension needed a 35-fold higher concentration of S2T1-6OTD (IC_{50}, 24.9 ± 3.1 μmol/L) compared with ss-Pu22 (IC_{50}, 0.70 ± 0.02 μmol/L; Fig. 1B and C), indicating much weaker binding and stabilization ability to G-quadruplex in ss-Telo24 than in ss-Pu22. This result was supported by CD spectroscopy, which showed that S2T1-6OTD failed to stabilize the conformation of ss-Telo24 to a G-quadruplex (Supplementary Fig. S2). Together, these results show a higher selectivity of S2T1-6OTD for the *c-Myc* promoter sequence compared with telomere DNA.

The binding affinity of S2T1-6OTD and stabilization of the G-quadruplex in the *c-Myc* promoter (c-MycQ) were further investigated by CD spectroscopy. Figure 2A shows that, under our experimental conditions, the *c-Myc* G-quadruplex–forming sequence exhibited the typical spectral signature of a G-quadruplex with a characteristic maximum at 264 nm (55, 56). In the presence of a five-times molar excess of S2T1-6OTD, the peak centered at 264 nm was significantly broader, with lower ellipticity

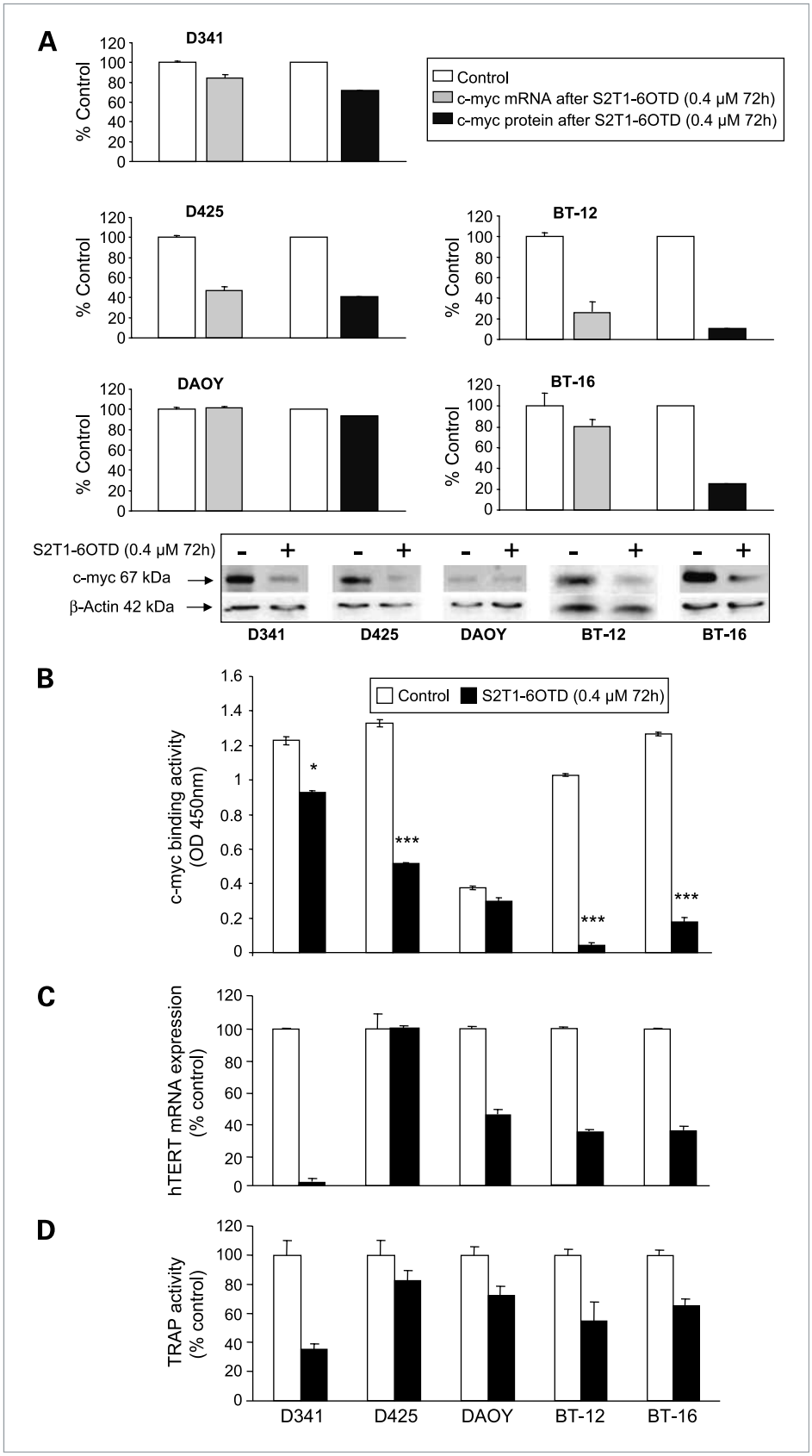

Figure 3. **A,** treatment with S2T1-6OTD results in a reduction of *c-Myc* mRNA as measured by quantitative real-time PCR ($n = 3$) and in a reduction of *c-Myc* protein expression (representative of two independent experiments) in D341, D425, BT-12, and BT-16 cells but not in DAOY cells. *Bars,* SD. **B,** treatment with S2T1-6OTD results in a significant reduction of *c-Myc* activity (measured by *c-Myc* binding capacity to its consensus sequence "CACGTG" of the E-box element) as determined by the ELISA-based TransAM *c-Myc* activity assay. *Columns,* mean absorbance ($n = 3$); *bars,* SD. *, $P < 0.05$; ***, $P < 0.001$. Treatment of MB and AT/RT cells with S2T1-6OTD resulted in a significant decrease of *hTERT* mRNA expression (**C**) and telomerase activity (**D**) as determined by quantitative real-time PCR and the Telomerase PCR ELISA kit. *Columns,* mean percentage decrease compared with untreated control cells ($n = 3$); *bars,* SD.

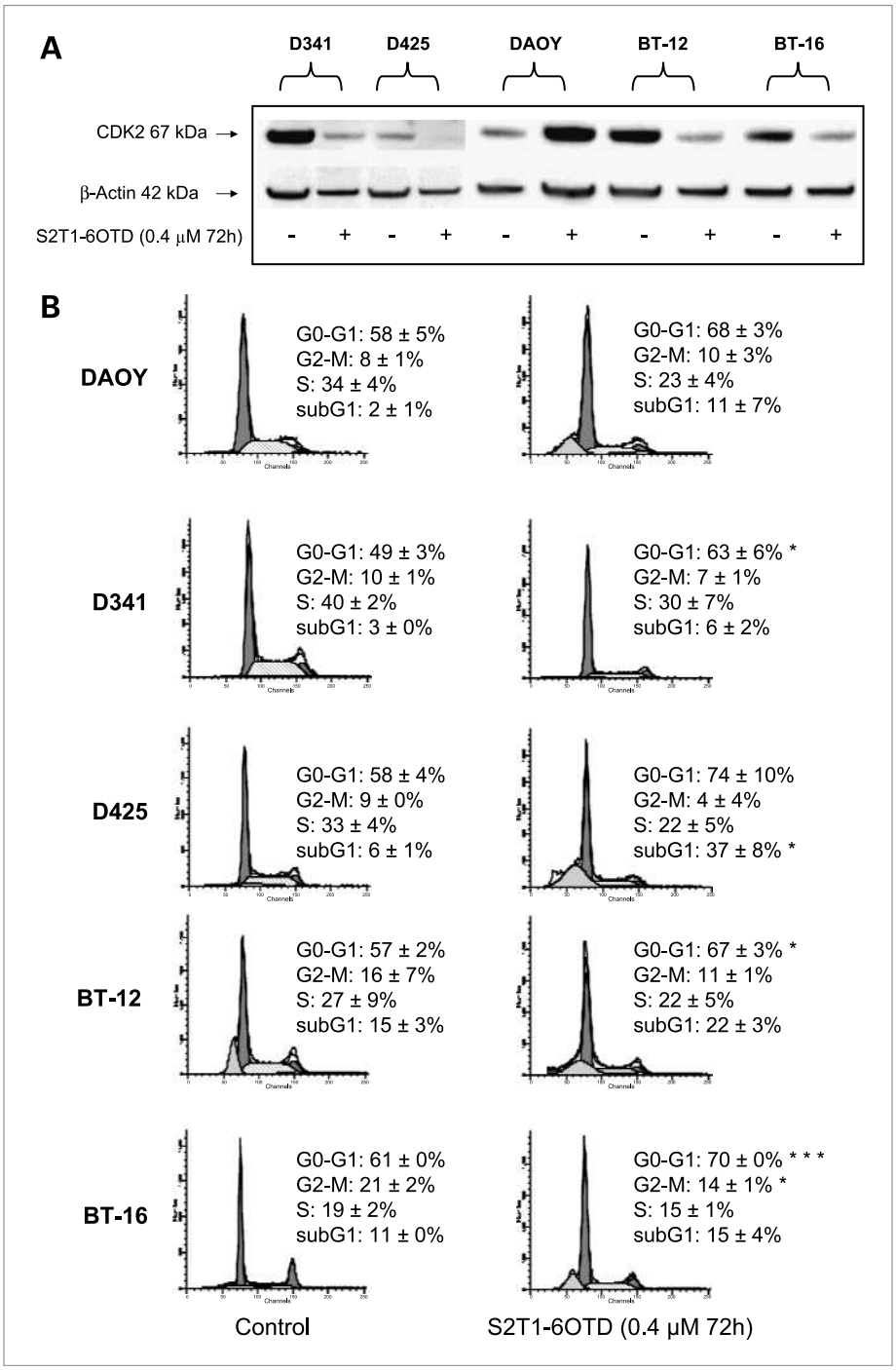

Figure 4. S2T1-6OTD reduces the protein expression of CDK2 and induces cell cycle arrest and apoptotic cell death in human MB and AT/RT cells. **A,** S2T1-6OTD decreased the cell cycle activator CDK2 protein expression in D341, D425, BT-12, and BT-16 but not in DAOY cells as determined by Western blotting. **B,** effects of S2T1-6OTD on cell cycle in human brain tumor cells as determined by fluorescence-activated cell sorting analysis. Cells were treated with S2T1-6OTD (0.4 μmol/L) or left untreated for 72 h and then fixed and stained as described in Materials and Methods. At least 20,000 cells were counted. Results are presented as percentages of cells in G_1, S, G_2-M, and sub-G_1 phases in two independent experiments. The subdiploid peak represents the apoptotic fraction. S2T1-6OTD treatment resulted in a decrease in the percentage of cells in the S phase and an increase in the cells in the G_0-G_1 and sub-G_1 phases in all cell lines tested. *, $P < 0.05$; ***, $P < 0.001$.

at the maximum. This indicates a physical interaction between S2T1-6OTD and the ssDNA of the *c-Myc* promoter sequence. However, the spectral differences were not large enough to permit titration experiments to calculate the binding constant.

Thermodynamic stability profiling provides information about the relative stability of DNA structures on ligand binding (57). Therefore, the stabilization effect of S2T1-6OTD on the G-quadruplex structure in the *c-Myc* promoter sequence was further studied by CD spectroscopy by measuring the thermodynamic stability profile of the ss-Pu22 oligomer and the change in absorption at 264 nm. The results in Fig. 2B show that the normalized CD intensity of ss-Pu22 at 264 nm with 50 μmol/L S2T1-6OTD in a Tris-HCl buffer (50 mmol/L, pH 7.4) had a T_m value of 46.7°C calculated from the CD melting curves at 264 nm using a sigmoidal fitting. Under the same conditions, the T_m value of S2T1-6OTD-Telo24 complex was

only 30.7°C (Fig. 2C), indicating a strong stabilizing effect of S2T1-6OTD on G-quadruplex in NHE III1 DNA and a weaker effect on G-quadruplex in telomere sequence.

Because indiscriminate binding of a compound to nonspecific DNA can result in significant loss of the compound and may have unintentional effects on the regulation of nontargeted genes, we investigated the binding ability of S2T1-6OTD to duplex DNA. A CD melting curve was done for the duplex ds-Pu22 in the presence or absence of 50 μmol/L S2T1-6OTD. The results showed no detectable change in melting temperature of the ds-Pu22 DNA in the presence or absence of S2T1-6OTD (Supplementary Fig. S3A), indicating the inability of S2T1-6OTD to bind to duplex DNAs. This result was confirmed by the electrophoretic mobility shift assay, which excluded the ability of S2T1-6OTD to interact with dsDNA sequences (Supplementary Fig. S3B).

S2T1-6OTD Treatment Down-Regulates *c-Myc* Expression and *c-Myc* Binding Activity

To test whether treatment with S2T1-6OTD alters c-Myc expression, we incubated a panel of MB and AT/RT cells with 0.4 μmol/L S2T1-6OTD for 72 hours and measured *c-Myc* mRNA expression by real-time quantitative reverse transcription-PCR and *c-Myc* protein by Western blotting. With the exception of DAOY cells (low basal *c-Myc* expression), treatment with S2T1-6OTD resulted in significant reductions of *c-Myc* mRNA and protein expression in all other MB and AT/RT cell lines tested (Fig. 3A). To examine whether treatment with S2T1-6OTD alters *c-Myc* binding activity, we incubated the five cell lines under study with S2T1-6OTD (0.4 μmol/L for 72 hours) and measured binding activity. S2T1-6OTD treatment reduced *c-Myc* binding activity significantly in all MB and AT/RT cells (Fig. 3B).

S2T1-6OTD Reduces Telomerase mRNA Expression and Activity

Because *hTERT*, the catalytic subunit of telomerase, is transcriptionally under the control of *c-Myc* (27, 28) and having shown an inhibitory effect of S2T1-6OTD on *c-Myc* expression in MB and AT/RT cells, we were interested in the effects that this interaction might have on telomerase activity. MB and AT/RT cells were treated with 0.4 μmol/L S2T1-6OTD for 72 hours, total RNA was isolated for reverse transcription-PCR, and total protein was extracted. Using real-time quantitative reverse transcription-PCR to measure *hTERT* mRNA and the Telomerase PCR ELISA kit to measure telomere repeat amplification protocol (TRAP) activity, it was found that S2T1-6OTD could indeed reduce *hTERT* mRNA expression in BT-12, BT-16, DAOY, and D341 cells. No effect was observed on *hTERT* mRNA in the mainly ALT (Alternative Lengthening of Telomeres)-dependent D425 cell line (Fig. 3C; ref. 47). Moreover, S2T1-6OTD reduced telomerase activity in all cell lines tested, with maximum inhibition in D341 (64%) and a minimum 17% in D425 (Fig. 3D).

The direct effect of S2T1-6OTD on telomerase activity was examined in a cell-free system. In this experiment, increasing concentrations of S2T1-6OTD (0.005–5 μmol/L) were added to the telomerase reaction mixture containing extract from D341 cells. The results showed that S2T1-6OTD inhibited *in vitro* the process of telomerase activity; the concentration that resulted in 50% decrease in telomerase inhibition (EC_{50}) was 0.1651 μmol/L when measured by TRAP and was 0.3577 μmol/L when measured by the TRAP-LIG assay (58), which was used to eliminate drug contamination of the PCR reaction. The results of these two experiments not only indicates that S2T1-6OTD by itself has no significant inhibitory effect on the PCR but also confirms that S2T1-6OTD possesses a direct effect on telomerase activity when examined in cell-free system (data not shown).

NHE III1 Sequence in the Promoter Region of *c-Myc* Is Needed for S2T1-6OTD to Exert Its Effect on *c-Myc*

To further confirm the requirement of the G-quadruplex–forming NHE III1 sequence in the promoter region of *c-Myc* for the exertion of downregulation of *c-Myc* expression by S2T1-6OTD, two Burkitt's lymphoma cell lines with different translocation break points within the *c-Myc* promoter (17, 59, 60) were investigated (Supplementary Fig. S4A). In the CA46 cells where the NHE III1 is deleted, S2T1-6OTD treatment (0.4 and 0.8 μmol/L for 72 hours) had no effect on *c-Myc* mRNA and protein expression, whereas in the Ramos cells, in which the NHE III1 is present, treatment with S2T1-6OTD significantly reduced *c-Myc* mRNA and protein expression (Supplementary Fig. S4B and C). In DAOY M2 MB cells where *c-Myc* is mainly under the control of a cytomegalovirus promoter (45), treatment with S2T1-6OTD did not decrease but rather slightly increased *c-Myc* expression (Supplementary Fig. S4D), also indicating a high affinity of S2T1-6OTD for NHE III1.

Effects of S2T1-6OTD on the Cell Cycle of MB and AT/RT Cells

To determine the possible effects of S2T1-6OTD on cell cycle regulation, we assessed the cellular DNA content by using flow cytometry (fluorescence-activated cell sorting) and determined CDK2 protein expression. With the exception of DAOY cells, S2T1-6OTD (0.4 μmol/L) treatment for 72 hours resulted in a significant decrease in the cell cycle activator CDK2 (Fig. 4A). Moreover, treatment with S2T1-6OTD resulted in a significant decrease in the percentage of cells that were able to enter the S phase and in an increase of the cells in the G_0-G_1 and sub-G_1 phases (Fig. 4B), indicating that S2T1-6OTD mediates cell cycle arrest and induction of apoptotic cell death.

S2T1-6OTD Treatment Results in Suppression of MB and AT/RT Cell Proliferation

To test whether treatment with S2T1-6OTD alters cellular proliferation, MB and AT/RT cells were incubated

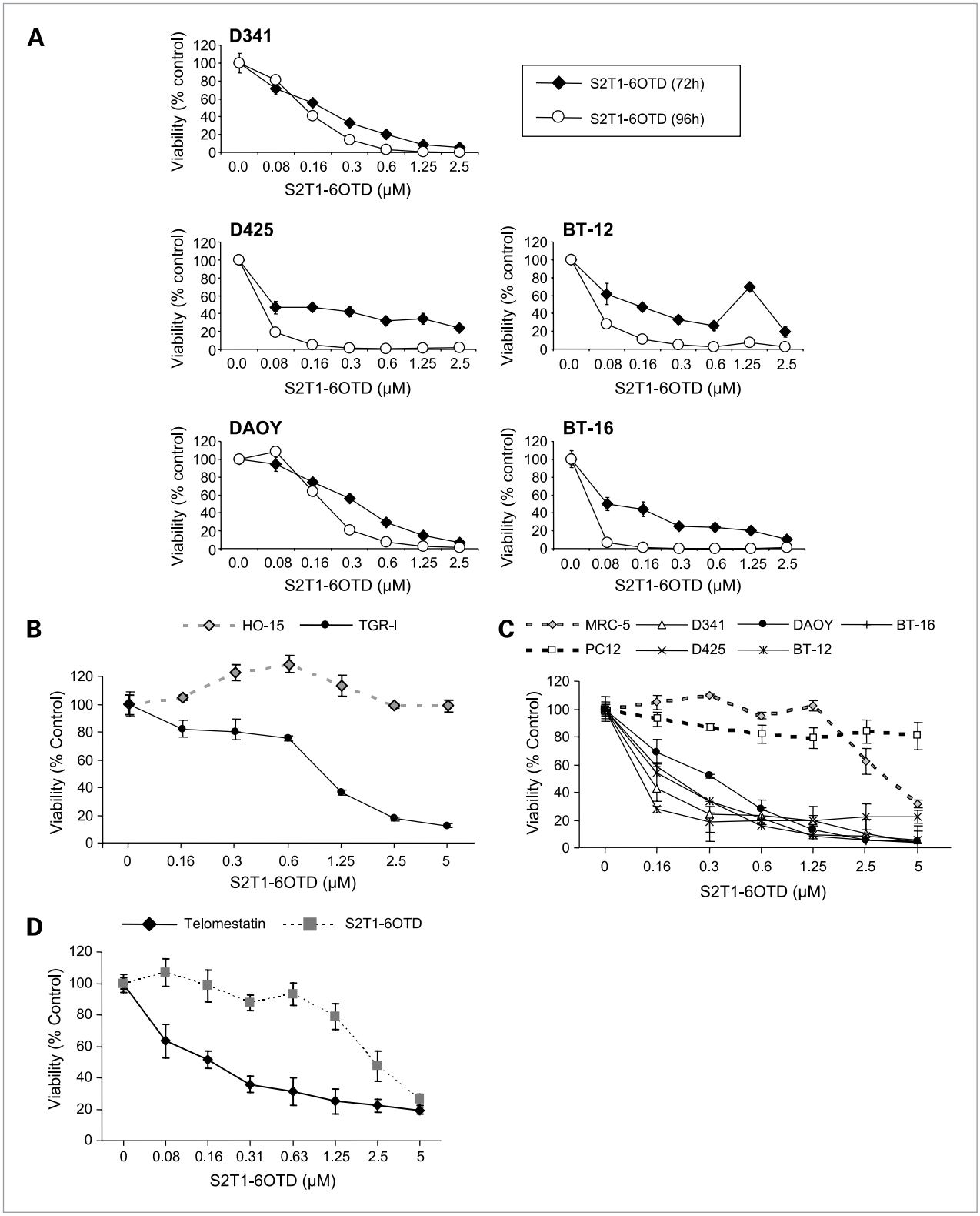

Figure 5. **A,** dose- and time-dependent inhibition of cell viability in MB and AT/RT cells by S2T1-6OTD as determined by MTS assay. **B,** S2T1-6OTD treatment had no antiproliferative effect on HO15.19 [*c-Myc* knockout (–/–)] rat fibroblast cells when compared with the parental TGR-1 *c-Myc* wild-type (rat fibroblast) cells. **C,** S2T1-6OTD treatment also had no major antiproliferative effect on PC12 cells (that do not depend on *c-Myc* in their proliferation) or MRC-5 normal fetal lung fibroblast cells when compared with MB and AT/RT cells. **D,** S2T1-6OTD exhibits much less cell toxicity than telomestatin on MRC-5.

with various concentrations of S2T1-6OTD for 72 or 96 hours and cell viability was assessed by using the MTS assay (Fig. 5A). Treatment with S2T1-6OTD resulted in a dose- and time-dependent cytotoxic response in all cell lines tested, with IC_{50} concentrations between 0.14 and 0.33 μmol/L (72-hour treatment time). However, S2T1-6OTD had no antiproliferative effect on *c-Myc* knockout (−/−) HO15.19 rat fibroblast cells when compared with the parental TGR-1 *c-Myc* wild-type (rat fibroblast) cells (Fig. 5B; ref. 61). Moreover, S2T1-6OTD also had no antiproliferative effect on PC12 cells. These cells do not depend on *c-Myc* to proliferate due to the absence of Max, the partner protein to which *c-Myc* dimerizes to be activated (Fig. 5C; ref. 62). When compared with brain tumor cells, S2T1-6OTD showed significantly less cytotoxic effects (IC_{50}, 3.44 μmol/L) on normal (not transformed) fetal lung fibroblast MRC-5 cells (63, 64), indicating some tumor specificity. Interestingly, when compared with the parental compound telomestatin, S2T1-6OTD showed much less cell toxicity on MRC-5 cells (Fig. 5D). Because S2T1-6OTD also causes a decrease in *hTERT* expression and activity, we investigated whether it was the *c-Myc* downregulation or the reduction in telomerase that exerted the antiproliferative effect of S2T1-6OTD on MB cell culture. To that end, we tested the effect of S2T1-6OTD on cell viability of telomerase-negative cells (e.g., the human foreskin fibroblasts BJ; ref. 65). Treatment with S2T1-6OTD resulted in a clear concentration- and time-dependent cytotoxic response in the telomerase-negative BJ cells (data not shown), indicating that telomerase is most likely not responsible for the effect of S2T1-6OTD on MB and AT/RT cell growth.

Effects of Long-term Treatment with Nontoxic Concentration of S2T1-6OTD

To examine the long-term effects of S2T1-6OTD on MB and AT/RT cells, cells were treated with nontoxic concentrations of S2T1-6OTD (0.04 or 0.004 μmol/L) for 5 weeks. We then characterized the growth properties of the treated cells during the long-term cultivation experiments. Treatment with S2T1-6OTD at 0.04 μmol/L resulted in telomere shortening in all MB and AT/RT cells tested (Fig. 6A). The shortening observed after 3 weeks of S2T1-6OTD treatment was 2.0 kbp in DAOY cells, 1.5 kbp in D341 cells, 2.6 kbp in BT-12 cells, 2.0 kbp in BT-16 cells, but only 0.8 kbp in D425 cells. The growth kinetics of S2T1-6OTD–treated MB and AT/RT cells did not differ significantly from those of untreated control cells in the first week. After 2 to 3 weeks, cell growth of S2T1-6OTD–treated cells decreased (Fig. 6B). Cells treated with S2T1-6OTD at 0.004 μmol/L were able to grow better as compared with cells treated with S2T1-6OTD at 0.04 μmol/L, but a decrease in cell growth was also observed with the very low concentration. The morphologic examination of the 0.04 μmol/L S2T1-6OTD–treated cells at the plateau phase showed an increased proportion of flat and giant cells with phenotypic characteristics of senescence and positive for senescence-associated β-galactosidase (data not shown). Finally, the cells underwent delayed apoptosis when examined morphologically (data not shown) and were characterized by an increase in apoptotic cell death, as measured by Cell Death Detection ELISA (Fig. 6C).

Discussion

In this study, we investigated the efficiency of S2T1-6OTD to stabilize G-quadruplexes in guanine-rich DNA and examined the strength of its selectivity for the *c-Myc* over the telomeric sequences. We then examined the S2T1-6OTD–mediated cellular effects in a representative set of childhood MB and AT/RT cells.

We have shown here that S2T1-6OTD binds more selectively with higher affinity to the G-quadruplex–forming sequence in the major transcription control element in the *c-Myc* promoter than to the telomeric DNA sequence. This high-affinity physical interaction was established by the results obtained from the PCR-stop assay, which showed that a 35-fold higher concentration of S2T1-6OTD is needed to stabilize a G-quadruplex structure in ss-Telo24 compared with ss-Pu22 sequence. This result was further confirmed by the CD spectroscopy experiments, which showed that the G-quadruplex structure stabilization by S2T1-6OTD in the *c-Myc* promoter tolerated a higher thermal melting point compared with the telomeric DNA sequence.

The preference of a compound for quadruplexes over duplex DNA is of great importance. This is because indiscriminate binding to a duplex DNA can result in a significant loss of the compound and might lead to unexpected cytotoxicity. Our results illustrated that S2T1-6OTD is unable to bind DNA duplexes, as shown by the absence of any changes in the thermal melting curve in the presence of S2T1-6OTD, compared with T_m of naked duplex DNA, rendering it suitable for further studies as a highly selective G-quadruplex–interacting molecule.

Through its ability to recognize and stabilize the quadruplex structure in the *c-Myc* promoter sequence, S2T1-6OTD was able to suppress both *c-Myc* mRNA and protein expression and reduce *c-Myc* activity in MB and AT/RT cell lines after 72-hour incubation; however, the ability to repress *c-Myc* expression was found to be cell type specific. Interestingly, when we treated MB DAOY M2 cells overexpressing *c-Myc* under a different promoter, S2T1-6OTD failed to downregulate c-Myc, indicating specific affinity to the transcriptional control element sequence NHE III1 in the *c-Myc* promoter. In agreement with this was our finding that S2T1-6OTD had no effect on *c-Myc* expression in Burkitt's lymphoma cell line CA46 with the deleted NHE III1 element due to its chromosomal aberration. These results therefore provided convincing evidence that the effect of S2T1-6OTD on *c-Myc* is indeed mediated through the interaction with the *c-Myc* promoter and further implicated effects on *c-Myc*–dependent downstream pathways. However, it has to be said that mechanisms regulating *c-Myc* transcription are multifaceted and involve

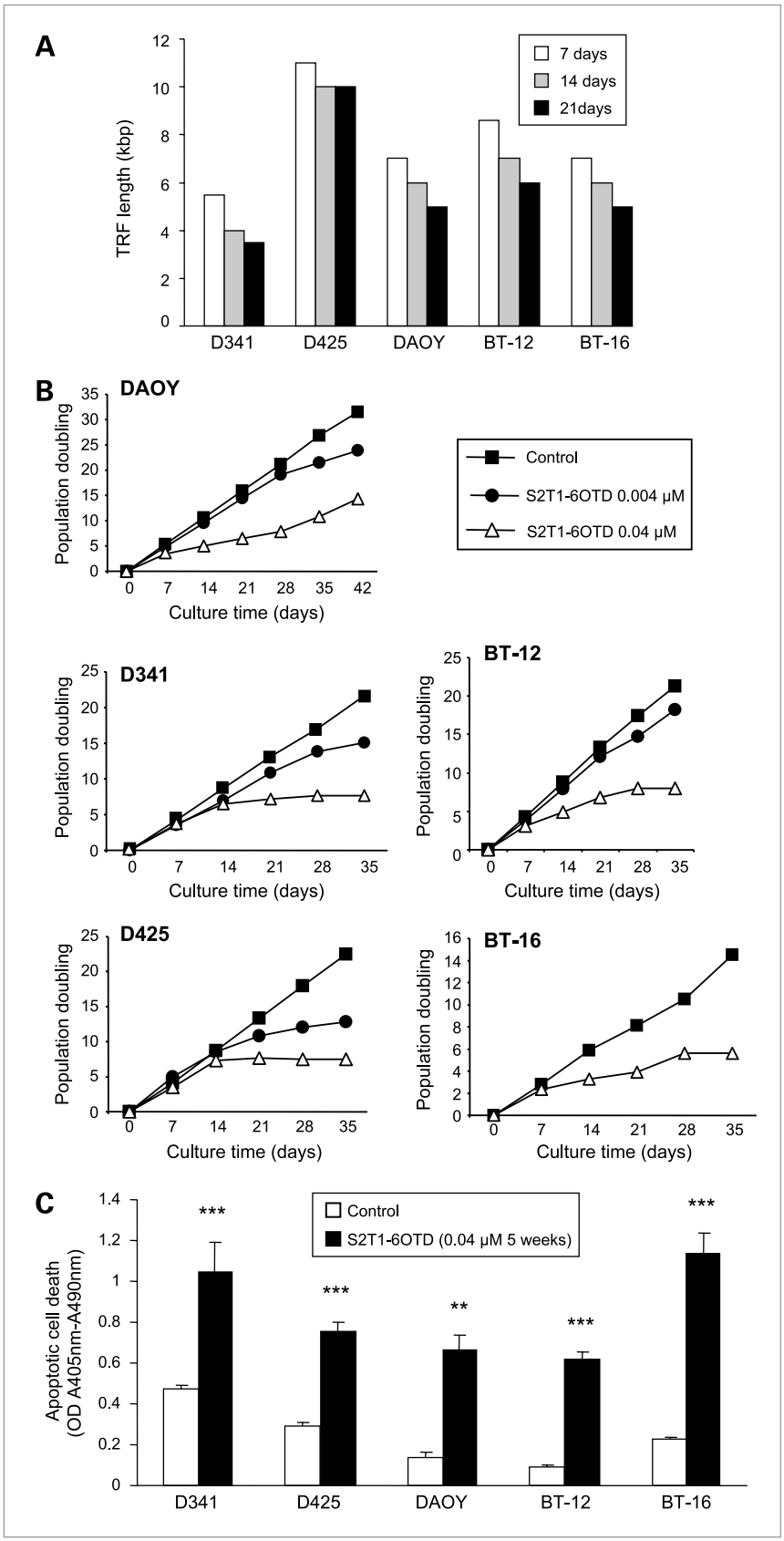

Figure 6. Effects of long-term treatments with nontoxic concentrations of S2T1-6OTD. Every 5 d, the cells were trypsinized and living cells were reseeded at the same number. **A,** effect of 0.04 μmol/L S2T1-6OTD on telomere length as determined by the TeloTAGGG Telomere Length Assay. All cell lines tested showed time-dependent telomere shortening. **B,** effect of 0.04 or 0.004 μmol/L S2T1-6OTD on cell growth as determined by MTS assay. All cell lines tested showed time-dependent decrease in cell growth. **C,** apoptotic cell death was quantified using Cell Death Detection ELISA measuring DNA fragmentation in S2T1-6OTD–treated cells (0.04 μmol/L, for 5 wk). *Columns,* mean absorbance of cytoplasmic histone-associated DNA fragments ($n = 3$; representative of two independent experiments); *bars,* SD. **, $P < 0.005$; ***, $P < 0.001$.

not only the regulatory DNA sequence in *c-Myc* promoter but also several DNA binding proteins that have been shown to preferentially bind to, stabilize, unwind, or cleave the *c-Myc* G-quadruplex structure (66–68). In addition, recent reports have shown that putative G-quadruplex sequences are widespread in >40% of human gene promoters (66, 67). Hence, it could be speculated that any mutation in the regulatory motifs, differences in DNA binding protein expression patterns, or off-target ligand interaction might be critical for the sensitivity toward G-quadruplex–based *c-Myc* inhibitor, such as S2T1-6OTD, in some cell lines, and their effect might present a different profile of biological activity than initially expected.

Based on previously published work, it is well established that the suppression of *c-Myc* expression is closely linked to the specific arrest in the G_0-G_1 cell cycle phase (69–72) and downregulation of *c-Myc* inhibits both cyclins and CDK2 expression in rhabdomyosarcoma (73), in T lymphocytes (74), and in colorectal cancer cells (75). Our results show that under the conditions where inhibition of both proliferation and *c-Myc* activity was observed, S2T1-6OTD treatment resulted in a decrease in the protein expression of the cell cycle activator CDK2 and in a clear G_0-G_1 cell cycle arrest in all brain tumor cell lines tested and decreased the percentage of the cells that were able to enter the S phase. This provided additional evidence that *c-Myc* must be a direct target for S2T1-6OTD action. However, it is noteworthy that S2T1-6OTD was able to induce cell cycle arrest despite no measurable decrease in the mRNA/protein level of *c-Myc* was observed in DAOY cells after 72-hour treatment. These results suggest that inhibition of *c-Myc* expression by S2T1-6OTD may not be the only mechanism explaining the G_0-G_1 arrest of the cell cycle and the cell growth inhibition, but the expression level of other oncogenes may be affected with an effect on downstream signaling pathways governing cell proliferation.

Many experimental lines of evidence from different groups have shown the association of *c-Myc* and cell proliferation (69, 76). We therefore expected that S2T1-6OTD treatments would induce a *c-Myc*–dependent cell growth arrest. Indeed, short-term treatments with S2T1-6OTD induced a dose- and time-dependent negative effect on cell viability of all the MB- and AT/RT-derived cell lines tested already at concentrations lower than 1 μmol/L. Of considerable interest was the finding that S2T1-6OTD showed a significantly lower antiproliferative effect when applied to normal human fibroblast cells MRC-5 (77). Ideally, a *c-Myc*–targeted therapy should be active on cells with deregulated *c-Myc* and should not affect the majority of the normal cells *in vivo*. Here, we showed a more prominent effect of S2T1-6OTD on cellular growth of MB- and AT/RT-derived cells compared with its weaker effects on normal cells, which would be supportive of a selective activity on neoplastic cells.

Treatment with S2T1-6OTD resulted not only in *c-Myc* downregulation but also in a decrease in *hTERT* mRNA and reduction of telomerase activity in most of the cell lines tested. This is of particular interest considering that the catalytic subunit of telomerase *hTERT* is transcriptionally regulated by *c-Myc* (27, 78) and that activation or repression of *c-Myc* can alter hTERT activity in normal or tumor cells both *in vitro* and *in vivo* (22, 28, 79–82). However, on the other hand, our finding that S2T1-6OTD binds more potently to the *c-Myc* promoter sequence did not exclude a weaker binding to telomeric DNA (with 35-fold less efficiency). Together with our discovery that S2T1-6OTD exerted a direct inhibitory effect on TRAP activity in a cell-free system, this result suggests a possible direct inhibition of the telomerase enzyme by S2T1-6OTD and could provide an explanation for the differences between the levels of *c-Myc* activity inhibition and the degree of hTERT activity reduction we observed in Fig. 4. Nevertheless, one should remember that S2T1-6OTD showed a dose- and time-dependent antiproliferative effect on the telomerase-negative human foreskin fibroblast cells (BJ) and on the ALT-dependent D425 while having no antiproliferative effect on *c-Myc* knockout (−/−) HO15.19 rat fibroblast cells or PC12 cells, which do not depend on *c-Myc* in their proliferation. Together, these data confirm that a functional *c-Myc* pathway, and not telomerase, is needed for S2T1-6OTD to exert its antiproliferative effect. This observation counters the concern about the specificity of S2T1-6OTD toward *c-Myc* and reveals that it is *c-Myc* inhibition rather than telomerase downregulation that is responsible for the proliferation arrest exerted by S2T1-6OTD in our experiments.

To extend our findings beyond the outcome of 72-hour treatment, we studied the effects of long-term treatment of MB and AT/RT cells with nontoxic concentrations of S2T1-6OTD, compatible with long-term drug dosage, and observed the effect of telomerase inhibition on the children's brain cancer cells. The desired effect of telomerase inhibition would be to shorten telomeres to critical lengths, causing replicative senescence and preferably cell death. The data obtained from our experiments showed that prolonged treatment did indeed result in a time-dependent telomere shortening that was preceded by a decrease in telomerase activity and accompanied by cell growth arrest and senescence-associated morphologic changes in MB and AT/RT cells, which also showed positive expression of senescence-associated markers. This finding was followed by apoptosis at day 35 in all cells treated.

In conclusion, we have shown the preferential recognition of the G-rich sequence in the *c-Myc* promoter over the telomere sequence by a novel small-molecule S2T1-6OTD. Through its ability to recognize and stabilize a G-quadruplex structure in the *c-Myc* promoter sequence, S2T1-6OTD was able to inhibit *c-Myc* and decrease telomerase activities, to disrupt telomere maintenance, to force cells into senescence, and to induce growth arrest and apoptosis in childhood brain tumor cell lines. A better knowledge about the *in vivo* pharmacokinetics and efficacy of S2T1-6OTD is mandatory. However, S2T1-6OTD may well represent a potential effective and innovative therapeutic strategy for childhood brain tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conclusions

Targeting MYC and its target genes as therapeutic strategy

MYC and its target genes have become attractive targets for cancer therapy, also due to MYC's correlation with poor prognosis and high-grade malignancy in different types of adult and pediatric tumors [37, 216-219]. Many attempts have been made so far to target MYC at different biological levels (discussed in appendix section, Review 1). Down-regulation of MYC may be achieved already at the transcriptional level, by either preventing the activation of the promoter [220] or destabilizing the specific transcript by antisense strategies. Small interference RNA (siRNA) molecules gave remarkable results in different tumor models, both *in vitro* and *in vivo*, and by this approach, MYC depletion led to a decrease of cell proliferation and inhibition of colony formation in soft agar assays, as well as a reduction in tumor growth in nude mice [221].

Although different therapeutic approaches have been evaluated to target MYC in various malignancies, such as MB [105, 216], direct pharmacological targeting of transcription factors has proven difficult. Moreover, designing MYC-selective therapeutic strategies must take into account the complexity of downstream signaling pathways, directly or indirectly depending on MYC, which can eventually lead to cellular responses with opposite effects. In fact, inhibiting MYC could affect its pro-apoptotic functions, which would be sensible to preserve in the context of anti-cancer therapies.

One of the major goals of our research was to investigate novel strategies to target the oncogene MYC and/or its target genes (cellular effectors) in the context of MYC-overexpressing ET and, in particular, in MB.

Quassinoids and NBT-272

While searching for molecules potentially useful as MYC inhibitors, we studied a group of compounds called quassinoids, historically known in folk medicine for their wide spectrum of activities (discussed in appendix section, Review 2). Quassinoids are heterogeneous molecules isolated from plants belonging to the *Simaroubaceae* family. Many members of this family are able to target the multiple signaling pathways and cellular processes that are necessary for a cancer cell to live. Together with cytotoxicity, protein synthesis inhibition, and induction of apoptosis, the capacity to down-regulate the oncogene MYC is a common feature among a subset of quassinoids [8, 16, 25, 27, 45]. We focused our research on a lipophilic semi-synthetic quassinoid analog named NBT-272. This compound showed the

ability to inhibit cellular proliferation of a variety of cancer cell lines and to trigger down-regulation of the MYC oncogene [222, 223]. The effects of NBT-272 have been investigated on different types of tumors where MYC plays an important role. In particular, it demonstrated a highly potent anti-proliferative effect when tested on *in vitro* models of NB and MB. Interestingly, cells with MYC amplification or over-expression appeared to be more sensitive and prone to apoptosis upon treatment. However, all cell lines tested were strongly affected, suggesting that MYC expression is not the only factor regulating cellular sensitivity to NBT-272 [222].

We deeply investigated the wide range of biological activities exerted by the quassinoid derivative NBT-272 on a panel of ET cell lines (discussed in result section, manuscript 2). Indeed, we were able to show that NBT-272 induces *in vitro* a complex pattern of cellular responses functionally related to interference with key cell survival pathways playing a role in the pathogenesis of several ETs (e.g., Akt/mTOR and Mek/Erk). Furthermore, by testing the compound in a xenograft model of human NB, we could confirm also *in vivo* that NBT-272 triggers down-regulation of the expression levels of MYC and reduction of the activation levels of Erk, causing an arrest of tumor growth.

In the search for an association between toxicity of NBT-272 and MYC expression levels in different ET cell lines, no significant correlation could be found. This observation led us to question any direct effect of NBT-272 on the oncogene, which would more likely be the result of different mechanisms, such as interference with protein synthesis and stability, also affecting the MYC family members, which are known to have short half-lives [224]. Indeed, we could observe that inhibition of the proteasome-dependent protein degradation pathway restored the depleting effect of NBT-272 and almost completely stabilized MYC. Moreover, depletion of MYC protein was associated with cell-cycle arrest and occurred via an indirect mechanism involving inhibition of two pro-proliferative pathways, i.e., the AKT and MEK/extracellular signal-regulated kinase (ERK) pathways. These two pathways converge to regulate the activation status of eIF4E and 4E-BP1, both dephosphorylated (i.e., inactive) in the presence of NBT-272, thus leading us to speculate that targeting these pathways would prevent full activation of the cap-dependent protein translation machinery.

Moreover, the effect induced by two specific MYC/MAX inhibitors [225, 226] and by *MYC* silencing in ET cells did not match the cellular response to NBT-272, again detrimental to the hypothesis of a direct effect on MYC. However, this conclusion does not exclude that MYC inhibition contributed to the cellular responses caused by NBT-272 treatment.

Our findings increase the present level of understanding of the mechanisms of action as anti-tumor agents of the quassinoids, and in particular of the lipophilic derivative NBT-272. However, the broad spectrum of biological effects exerted by the quassinoid compounds highlights a need for more accurate and systematic screening of these molecules as well as for a rational drug design-based study to identify leads for the synthesis or semi-synthesis of new analogs with increased activity, decreased toxicity and/or improved pharmacological profiles for the development of future cancer therapeutics.

Disabling MYC by a G-quadruplex interactive agent

Our research investigation focused on a different approach for targeting MYC acting through the stabilization of the quadruplex structure in the promoter sequence of the oncogene by the compound S2T1-6OTD (discussed in result section, manuscript 3). S2T1-6OTD is a molecule able to suppress both mRNA and protein expression of MYC. We examined the efficiency of this compound to stabilize G-quadruplexes in guanine-rich DNA and the strength of its selectivity for MYC over the telomeric sequences.

Moreover, we studied the S2T1-6OTD-mediated cellular effects in a representative set of childhood MB and atypical teratoid/rhabdoid tumor (AT/RT) cells. S2T1-6OTD induced a dose- and time-dependent negative effect on cell viability of all tumor cell lines tested and it reduced the activity of MYC in a cell-type specific manner. Recent reports have shown that putative G-quadruplex sequences are widespread in >40% of human gene promoters (66, 67). Hence, it could be speculated that any mutation in regulatory motifs, differences in DNA-binding protein expression patterns, or off-target ligand interaction might be critical for sensitivity to G-quadruplex-based MYC inhibitors, such as S2T1-6OTD, in some cell lines. Furthermore, their effect might present a different profile of biological activity than initially expected.

Our results show that in parallel to the inhibition of both proliferation and MYC activity, S2T1-6OTD treatment provoked a decrease in the protein expression of the cell-cycle activator *CDK2* and a G_0-G_1 cell-cycle arrest in all brain tumor cell lines tested. This provided additional evidence that MYC must be a direct target for S2T1-6OTD action. However, it is noteworthy that S2T1-6OTD was able to induce cell-cycle arrest despite no measurable decrease in the mRNA/protein level of MYC was observed. These results suggest that inhibition of the oncogene expression may not be the only mechanism explaining the G_0-G_1 cell-cycle arrest and cell growth inhibition.

Moreover, through its ability to recognize and stabilize a G-quadruplex structure in the promoter sequence of MYC, S2T1-6OTD was also able to decrease telomerase activities, to disrupt telomere maintenance, to force cells into senescence, and to induce growth arrest and apoptosis in childhood brain tumor cell lines. Thus, S2T1-6OTD may represent a potential effective and innovative therapeutic strategy for childhood brain tumors. However, better knowledge of *in vivo* pharmacokinetics and efficacy of this compound is still needed.

MYC target genes candidates in MB

There is growing evidence that a subset of MB tumors derives from an aberrant development of GNP cells triggered and sustained by a deregulated expression of the cellular pathways physiologically active during embryogenesis. Although MYC does not represent a pathway "per se", the oncogene is a central mediator of virtually all the pathways known to take part in cerebellum development and found altered in MB, including the BMP/SMAD pathway.

Better knowledge of the MYC effector genes involved in MB neoplastic transformation is of great relevance in order to find novel and suitable therapeutic strategies. We defined a list of candidate MYC target genes with potential roles in MB onset and/or progression. Our instrumental approach to investigate MYC-regulated genes consisted of using a model of MB-derived cells and profiling them by cDNA microarray upon genetic manipulation aiming at either overexpressing [37] or down-regulating *MYC*. The resulting sets of differentially expressed genes were then intersected to narrow down the great amount of data generated and to finally define a group of MYC-responsive genes. The list of 209 candidates comprises 105 genes up-regulated and 104 genes down-regulated by *MYC* overexpression, and consistently following the levels of the oncogene upon *MYC* silencing. These genes included known direct targets such as *CCND2* [112] and *TERT* [227]. In fact, over-expression of *MYC* increased the *CCND2* level by 3.1- and 2.3-fold in DAOY M2.1 and M14 cells, respectively, whereas *MYC* silencing repressed the same gene by 8.4-fold. In contrast, a gene that is known to be negatively regulated by MYC, inhibin-beta A (*INHBA*), was significantly downregulated in DAOY M2.1 and DAOY M14 cells (3.8- and 3.7-fold change, respectively) and upregulated upon MYC silencing (3.9-fold change).

Novel candidate target genes of potential interest have been evaluated. In particular, gene ontology analysis led us to identify components of pathways which were already known to play a role in the onset and progression of MB but had not been correlated with MYC and which may play a relevant role in MYC-driven cellular responses (discussed in result section, manuscript 1).

BMP7 is a MYC target with pro-survival functions in MB

The gene expression analysis we performed brought to our attention components of the BMP pathway that are responsive to MYC modulation in MB cells (discussed in result section, manuscript 1). In particular, a general increase in the mRNA expression levels of BMP ligands (*BMP2*, *6*, *7*) in DAOY M2.1 cells hinted at MYC-dependent activation. Many subtypes of BMP receptors (BMPR) type I-II were also represented. However, only few of them could be correlated in expression with MYC in our cellular models. Similar to the majority of BMPs, the mRNA level of glypican-3 (*GPC3*), a soluble inhibitor of BMPs [228, 229], increased with MYC up-regulation, and it may be responsible for a negative-feedback loop on the pathway. Our microarray data did not show significant alteration of members of the SMAD family of transcription factors or of the SMAD target genes inhibitors of differentiation (ID) [230]. This observation would imply the involvement of alternative downstream effectors, which are yet to be fully characterized.

Among the BMP members modulated by MYC, *BMP7* was the only one presenting consistently induced/repressed high fold-change values along with *MYC* up-/down-regulation, respectively.

In this context, recent evidences suggest that BMP7 contributes to determining a correct temporal regulation and spatial organization of cerebellar progenitor cells during development [40, 69]. Along with this role, deregulated BMP7 expression in the cerebellum is very likely to trigger abnormal cell development and tumorigenesis.

Our investigation shows for the first time the existence of a functional link between *MYC* over-expression and abnormal BMP pathway regulation in a model of MB.

That *BMP7* is a direct target of MYC was previously reported by Zeller et al. [113], who performed a global mapping of MYC-binding genomic regions in a model of human B lymphoid tumor. *BMP4*, another BMP family member, was described to be a target of MYC in blood cells [231]. However, these reports represent the only experimental evidence, to our knowledge, of a transcriptional control of the BMP pathway by the MYC oncoprotein. More specifically, no previous studies have mentioned MYC-driven regulation of this pathway as a significant molecular network in MB. We proved that *BMP7* is a direct MYC target showing the binding of MYC to the promoter of *BMP7* by ChIP-on-chip analysis in different MB cell lines.

Furthermore, BMP7 was analyzed by IHC in primary MB samples. Strikingly, BMP7 was not expressed in normal cerebellum, indicating that BMP7 over-expression in MB may

contribute to the increased proliferation and survival of tumor cells. Moreover, we found a significant correlation between the gene expression of MYC and BMP7 in two distinct datasets of MB patient samples.

The functional relevance of the BMP-dependent signaling pathway in MB was evaluated by targeting the BMP pathway via specific siRNA-mediated silencing of *BMP7* expression and employing a small-molecule inhibitor (Dorsomorphin) able to prevent the activation of SMAD1/5/8 by interfering with the kinase activity of the receptors BMPR-I/II [232, 233]. Conversely, DM has no, or very limited, effect on other components of the TGF-β family (e.g., TGF-β1 and activin A) [233]. DM strongly impaired cell proliferation and viability, and interestingly, cells expressing a single copy, or low levels, of *MYC* were significantly less sensitive to DM than cells overexpressing the oncogene, indicating that the compound can affect a MYC-driven pro-survival response. Similarly, the silencing of *BMP7* alone induced cell death and inhibition of cell viability, even though it had a less prominent effect compared to the toxicity of DM. This observation is reasonable because the compound can block the pathway more efficiently by acting on more components than single siRNAs.

Therefore, our results show the feasibility and potential benefit of selectively blocking the BMP/SMAD pathway by either gene silencing or small-molecule inhibitors – strategies that were investigated here for the first time in MB cells. A therapeutic approach preventing deregulated activation of this pathway in MB would certainly represent one additional attractive strategy to be considered, together with the interference of SHH and WNT pathways, for which therapeutic targets have been already described [234-237]. However, as a novel therapeutic approach for pediatric malignancies, this strategy should be carefully evaluated for its efficacy and safety. Additional investigations about the other members of the BMP family with potential roles in MB are needed, since the available studies are often controversial (i.e., treatment of MB cells with BMP2 and BMP4 showed either attenuation of intrinsic apoptotic mechanisms and increase in cell count [214] or inhibition of proliferation [215]).

In conclusion, our results provide evidence of the induction of BMP7 as a MYC-dependent mechanism and indicate this growth factor as a direct target of MYC and a potential effector protein during MYC-driven neoplastic transformation of MB precursor cells. This new information not only contributes to a better understanding of the pro-survival functions of MYC during MB development, but it also suggests a rationale for targeting BMP pathways as a therapeutic intervention strategy for MB patients with MYC amplification/overexpression.

An extensive *in vivo* analysis is required to significantly demonstrate the correlation between *MYC* and *BMP7* and to have a better insight about their actual roles in MB tumorigenicity.

Curriculum Vitae

curriculum vitae

Personal information

Surname / First name	**Fiaschetti Giulio**
Address	Am Suteracher 37, 8048 Zurich, Switzerland
Mobile telephone	+41 (0)76 507 6763
E-mails	Giulio.Fiaschetti@kispi.uzh.ch ; giuliofiaschetti@hotmail.com
Nationality	Italian
Date of birth	07. 06.1979

Work experience

Dates	February 2007 - April 2011
Position held	**PhD University of Zurich, Kinderspital, Pediatric Oncology Division** (University Children's Hospital, Steinwiesstrasse 75. CH-8032 Zurich, Switzerland)
Name and address of employer	Prof. Dr. Michael Grotzer, Head of Pediatric Neuro-Oncology / Oncology Tel: +41 44 266 7575 e-mail: Michael.Grotzer@kispi.uzh.ch (University Children's Hospital, Steinwiesstrasse 75. CH-8032 Zurich, Switzerland) Supervisor: PD Dr. Alexandre Arcaro, Universität Bern, Departement Klinische Forschung, Tiefenaustrasse 120c, 3004 Bern, Switzerland Tel.+41 31 308 80 29 e-mail alexandre.arcaro@dkf.unibe.ch Thesis Committee: Prof. Dr. Martin Pruschy, Laboratory for Molecular Radiobiology, Department of Radiation Oncology, University Hospital Zürich, Tel: +41-44-255 85 49 e-mail: martin.pruschy@usz.ch Nathan W. Luedtke, Assistant Professor, Universität Zürich Organisch-chemisches Institut, Tel: +41 44 635 4244 e-mail: luedtke@oci.unizh.ch

Education and training

Dates	June 2004- July 2006
Title of qualification awarded	**MSc in Pharmaceutical Chemistry and Technologies** Department of Human Physiology and Pharmacology, University of Rome "La Sapienza" (IT) **Degree in Pharmaceutical Chemistry and Technologies** University of Rome "La Sapienza" (IT) Grade: 106/110
Principal subjects	Master thesis: "Growth inhibition and cell cycle arrest effects of *Cistus incanus* L. extract on three selected human prostate tumor cell lines". Dr. Luciano Saso, Department of Human Physiology and Pharmacology, University of Rome "La Sapienza" (IT) e-mail: luciano.saso@uniroma1.it Dr. Beatrice Tita, Department of Human Physiology and Pharmacology, University of Rome "La Sapienza" (IT) e-mail: beatrice.tita@uniroma1.it

Personal skills and competences

Mother tongue: **Italian**

Other languages:

English	Spanish	Deutsch
Fluent	Good	Good

Technical skills and competences

Cell culture methods (monolayer and suspension cultures); Cell proliferation, cytotoxicity and cell viability (MTT, MTS, BrdU incorporation, SRB, Trypan blue); Cell survival (Cloning in monolayer and in soft agar); Anchorage independent Growth (Soft Agar Clonogenic Assay); Cell cycle analysis (Fluorescent activated cell sorting).

Molecular Biology: PCR; RT-PCR; Sequencing; Nucleic acids (DNA, RNA, miRNA) extraction, isolation and analysis; Transfection (siRNA, shRNA, miRNA)

Biochemistry: Purification of proteins; Immunoprecipitation; Protein quantification; Western blot analysis; Dosage of cytochines by ELISA method; Chromatin Immunoprecipitation (ChIP)

Computer skills and competences

Windows: MS Office (Word, PowerPoint, Picture Manager, Excel, Outlook).

Image optimization and analysis: Picture Manager, Photoshop.

Tools for Molecular Biology: Applied Biosystem SDS 2.3 - RQ Manager; DAVID; GENEGO; Ingenuity Pathway Analysis; SigmaPlot 10.0; Endnote/Reference Manager. Data mining in Pubmed and NCBI sequence databases.

Post Graduation courses

Molecular and cell biology of Cancer (2010)

Clinical Cancer Research Course (2009)

Scientific Writing Course (2009)

LTK1 Module 1E: Introductory Course in Laboratory Animal Science (2008)

Publications

Bone morphogenetic protein-7 as novel c-Myc target gene in childhood medulloblastoma
Giulio Fiaschetti, Deborah Castelletti, Stefan Zoller, Alexander Schramm, Christina Schroeder, Masaya Nagaishi, Duncan Stearns, Michael Mittelbronn, Angelika Eggert, Frank Westermann, Hiroko Ohgaki, Tarek Shalaby, Martin Pruschy, Alexandre Arcaro, Michael A. Grotzer.
Oncogene (Submitted, December 2010)

Quassinoids: from traditional drugs to new cancer therapeutics
Giulio Fiaschetti, Michael A. Grotzer, Tarek Shalaby, Deborah Castelletti, Alexandre Arcaro
Current Medicinal Chemistry (Accepted, November 2010). Review.

The quassinoid derivative NBT-272 targets both the mTOR and Erk signaling pathways in embryonal tumors
Deborah Castelletti, Giulio Fiaschetti, Valeria Di Dato, Urs Ziegler, Candy Kumps, Katleen De Preter, Frank Westermann, Massimo Zollo, Frank Speleman, Tarek Shalaby, Daniela De Martino, T. Berg, Angelika Eggert, Alexandre Arcaro, and Michael A. Grotzer.
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Disabling *c-Myc* in Childhood Medulloblastoma and Atypical Teratoid/Rhabdoid Tumor Cells by the Potent G-Quadruplex Interactive Agent S2T1-6OTD
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Grotzer MA, Castelletti D, Fiaschetti G, Shalaby T, Arcaro A.
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miR-21 Suppression Impedes Medulloblastoma Cell Migration
Grunder E1*, D'Ambrosio R2*, Fiaschetti G*1, Abela L1, Arcaro A2, Zuzak TJ3, Ohgaki H4, Lv SQ,4,5, Shalaby T1, Grotzer M1
* Contributed equally to this work.
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Genetic alterations of microRNA potentially targeting MYCC and the role of miR512-2 in medulloblastomas
Sheng-Qing Lv[1,2], Young-Ho Kim[1], Fiaschetti Giulio[2], Shalaby Tarek[2], Sumihito Nubusawa[1,4], Hui Yang[3], Zheng Zhou[3], Grotzer Michael[2], Hiroko Ohgaki[1]
[Submitted; Molecular Cancer Therapeutics; April 2011]

Talks

European Embryonal Tumor Pipeline Meeting
Ghent, 24 -26 November 2009 - SMAD and NOTCH pathways in Medulloblastoma As potential c-Myc targets

European Embryonal Tumor Pipeline Meeting
Vienna, 22-25March 2009 - Identification and validation of c-Myc target genes related to cell cycle in Medulloblastoma

European Embryonal Tumor Pipeline Meeting
Ljubljana (Slovenia), 21-24 June 2008 - Identification and validation of c-Myc target genes related to cell cycle in Medulloblastoma

Poster presentations

Charles Rodolphe Brupbacher; Zurich, Februar 2011; "Bone morphogenetic protein 7 is a MYC target with pro-survival functions in childhood medulloblastoma"

KISPI Zürich Posterpräsentation; 21 and 28 April 2009; Identification and validation of novel c-Myc target genes in medulloblastoma.

Charles Rodolphe Brupbacher Stiftung "Targets for Cancer Prevention and Therapy"; Zurich, 11-13 Februar 2009 - Anti-proliferative effect of the Myc-inhibitor NBT-272 in embryonal tumor-derived cell lines

BACR –The Royal Society of Medicine "Cell signaling and novel cancer therapeutics"; London, 29-30 Nov 2007 - Anti-proliferative effect of the Myc-inhibitor NBT-272 in embryonal tumor-derived cell lines

Abstracts

Expression profile of Myc family genes and preclinical testing of NBT-272 in E.T.-derived cell lines. D. Castelletti, G. Fiaschetti, A. Arcaro, and M. Grotzer. Embryonal Tumor Pipeline - Cagliari (Italy), 10-11 September 2007

Growth Reduction, Apoptosis Induction and Telomere Maintenance Disruption in Medulloblastoma and Teratoid/Rhabdoid CNS Tumors Cells Treated by Novel G-Quadruplex Interactive Agent. T. Shalaby, A. Von Büren a, ML Hürlimann a, G. Fiaschetti a, K. Shin-ya b, K. Nagasawa b, A. Arcaro, M. A. Grotzer. SGP Annual Meeting. 14. /15. June 2007, Zurich.

Targeting Myc in Embryonal Tumors. Anti-proliferative effect of NBT-272 in embryonal tumor-cell lines. D. Castelletti, G. Fiaschetti, B. Fischer, A.O. von Büren, T. Shalaby, A. Arcaro, and M.A. Grotzer. Schweizer Forschertag Pädiatrie - Bern 13th November 2007

Disabling the proliferative and replicative ability of Medulloblastoma and Teratoid/Rhabdoid CNS Tumor Cells by a novel Telomestatin derivative. T. Shalaby, A. von Büren, M.L. Hürlimann, G. Fiaschetti, D. Castelletti, K. Shin-ya, K. Nagasawa, A. Arcaro, M. A. Grotzer. Wissenschaftliche Tagung der SPOG 25./26. Januar 2008, Locarno Symposium on Pediatric Neuro-Oncology 2008, Chicago, Illinois, USA on June 29 - July 2, 2008 (submitted)

Driving licence

A B European driving licence

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Targeting Myc in Pediatric Malignancies of the Central and Peripheral Nervous System

Michael A. Grotzer*[,#], Deborah Castelletti[#], Giulio Fiaschetti, Tarek Shalaby and Alexandre Arcaro

Department of Oncology, University Children's Hospital Zurich, Switzerland

Abstract: *Myc* family genes are often deregulated in embryonal tumors of childhood including medulloblastoma and neuroblastoma and are frequently associated with aggressive, poorly differentiated tumors. The Myc protein is a transcription factor that regulates a variety of cellular processes including cell growth and proliferation, cell cycle progression, transcription, differentiation, apoptosis, and cell motility. Potential strategies that either inhibit the proliferation-promoting effect of Myc and/or activate its pro-apoptotic function are presently being explored. In this review, we will give an overview of Myc activation in embryonal tumors and discuss current strategies aimed at targeting Myc for cancer treatment.

Keywords: Cancer, child, brain tumor, neuroblastoma, targeted therapies, Myc.

INTRODUCTION

Embryonal Tumors of the Central and Peripheral Nervous System

Embryonal tumors of the central and peripheral nervous system including medulloblastoma (MB), atypical teratoid/rhabdoid tumors (AT/RT), and neuroblastoma (NB) occur most commonly in the first few years of life and account for approximately 20% of childhood malignancies [1]. This broad group of childhood tumors originates from immature tissue as a result of the aberrant proliferation of early precursor cells and their morphological appearance resembles that of tissues in the developing embryo and fetus [2, 3]. Tumors of the central nervous system have been repetitively re-classified by the World Health Organization (WHO) [4, 5], along with the acquisition of further knowledge about their biology. One of the biggest controversies was how to describe the four MB variants, discriminating them from other types of primitive neuroectodermal tumors (PNET) [6-8]. The continuous nomenclature changes also reflect the improved understanding of the signaling pathways and molecular processes involved in tumorigenesis at different levels (i.e. genetic, transcriptional, and protein level), leading to definite steps forward towards a more accurate patients stratification and the development of novel therapeutic strategies.

During embryonal development, tissue growth and differentiation are regulated by complex cellular processes that involve precise control of both cell division and apoptosis. Defects in any of the pathways controlling these events can promote transformation, making these developing cells particularly prone to tumorigenesis [9]. Since its identification as the cellular homologue of the avian myelocytomatosis virus oncogene (v-myc), the human *myc* family of genes (*c-myc*, *MYCN*, *L-myc*) has emerged as a critical regulator of embryonal cell growth and one of the most frequently altered in cancer [10-13].

*Address correspondence to this author at the University Children's Hospital Zurich, Department of Oncology, Steinwiesstrasse 75, CH-8032 Zurich, Switzerland; Tel: +41 44 266 7575; Fax: +41 44 266 7171; E-mail: Michael.Grotzer@kispi.uzh.ch

[#]These authors contributed equally

Myc in Normal and Cancer Cells

Myc is broadly expressed during mammalian development and is detectable at the RNA level in various embryonal tissues [14, 15]. In mouse, the expression of the *myc* genes is differentially regulated during embryogenesis, with tissue- and cell-specific patterns of expression unique to *MYCN* as compared to the related *c-myc* gene [16]. In particular, *MYCN* shows the highest mRNA expression in embryonal brain, kidney, and lung [17, 18], whereas *c-myc* levels are comparatively constant in these organs throughout development [19, 20]. Knockout of either of these two *myc* genes resulted to be lethal. The mouse embryos died in the earliest developmental phases [21-23], confirming the essential role of the proto-oncogene in the proliferation control during mammalian embryogenesis. On the other hand however, the other family member L-myc seems to be dispensable, since *L-myc* -/- mouse embryos were fully viable and had a normal appearance [24].

Myc proteins are transcription factors with a basic-helix-loop-helix-leucine zipper (B-HLH-LZ) motif, responsible for binding to DNA and for dimerization with the partner protein Max, following upstream activating signals [25]. Myc/Max heterodimers can bind to the consensus sequence *CACGTG* of E-box promoter elements and recruit elongation factors, ATPases, histone acetyltransferases, and the E3 ubiquitin ligase Skp2, in order to promote gene transcription [26]. Alternatively, gene expression can also be repressed by the involvement of additional Myc-interacting factors, such as Miz1 [26], Sp1 [27], and YY1 [28]. Many Myc target genes have been already identified by using different experimental approaches, including cDNA microarray analysis and chromatin immunoprecipitation [25, 29-33]. These studies revealed that Myc is a multifunctional transcription factor, determining the expression of a large number of downstream target genes that are the actual effectors of the diverse Myc-dependent cellular responses. The same cellular functions can be deregulated during tumorigenesis and play a role in cancer development: cell cycle progression [34-37], cell proliferation [26, 38-40], apoptosis [41-45], immortalization [46, 47], angiogenesis [22, 48], and cellular differentiation [49, 50].

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In quiescent adult cells, Myc is almost undetectable [51]. Activation of Myc can be driven by growth factors, mitogens, and other transcription factors like β-catenin, enhancing the transcription rate of the *myc* gene [52]. Alternatively, Myc expression can be suppressed by transforming growth factor-β (TGF-β) and by an auto-regulatory mechanism [53, 54]. Upon mitogenic stimulation, the mRNA and protein levels of Myc increase rapidly and determine the beginning of the G1 phase of the cell cycle. This event is transient and followed by a gradual decline of Myc expression to low, but still detectable, steady-state levels in proliferating cells [51, 55, 56].

In contrast to the strictly regulated expression of the *myc* genes during normal cellular proliferation, cancer cells may activate *myc* in an uncontrolled fashion, as a result of genetic aberrations, therefore no longer requiring exogenous stimuli from the microenvironment to proliferate [57]. *Myc* can be abnormally activated by gene amplification [12, 58, 59], chromosomal translocation and DNA rearrangement [60-62], upregulation of transcription [52], and point mutation [63, 64], all leading to a constitutive overexpression of the oncogene. Sustained high levels of Myc seem to be required for cell survival and acquisition of a malignant phenotype by cells of different origin, e.g. hematopoietic cells [65], mammary glands [66], hepatic [67] and epithelial cells [68], as well as pancreatic islets [69]. However, besides the Burkitt's lymphoma [70] and the myeloid and plasma cell leukemia [71], deregulation of Myc represents a malignant signature for many more types of cancer, such as breast carcinoma [72, 73], ovarian carcinoma [74, 75], colon carcinoma [76], small cell lung carcinoma [77], and neuroectodermal tumors [2, 3]. In many of these neoplasias the overexpression of Myc has been associated with tumor aggressiveness and low cellular differentiation, although the molecular mechanisms underlying Myc-induced tumorigenesis are not completely understood yet [78].

Transgenic Mouse Models

The ability of Myc to transform cells *in vitro* and to promote cell proliferation and arrest of cellular differentiation represent strong evidences supporting the role of Myc in tumorigenesis [79]. Additionally, the development of transgenic animals with inducible and tunable expression of the oncogene further proved the transforming potential of Myc *in vivo*. Transgenic mice expressing *MYCN* under the control of the rat tyrosine hydroxylase (TH) promoter [80] have been proven to be a good model of NB, because of tumor formation in peripheral neural crest-derived structures showing histological features that resemble the human disease [80, 81].

B-cell lymphoma was induced in a different mouse model by overexpressing *c-myc* under the control of the immunoglobulin heavy chain enhancer (Eμ) [82], unrevealing important c-Myc-mediated mechanisms of tumorigenesis that involve downstream regulators of apoptosis. The authors showed that Myc overexpression in Eμ-myc mice (model for the human non-Hodgkin's lymphoma) selects for loss of p19(ARF) and/or p53 function and therefore inactivates the ARF-MDM2-p53 pro-apoptotic pathway *in vivo* [82]. Alternatively, the anti-apoptotic proteins Bcl-2 and Bcl-X_L can be overexpressed as a result of Myc upregulation, representing an additional distinct mechanism of resistance to apoptosis triggered by the oncogene [83, 84]. The same mouse model also allowed further studies on the effect of upregulation of MDM2 in B cells. It has been shown that MDM2 can mediate genomic instability and prevent p53-dependent apoptosis [85, 86], suggesting MDM2 as a tumor target.

Taken together, the clinical and experimental observations collected so far warrant further efforts aimed at better understanding the biological role of Myc in tumorigenesis with the perspective of developing effective Myc-targeted therapeutic strategies for cancer treatment [87-89]. The ultimate goal is the development of more efficacious and less toxic treatment schedules, used alone or combined with conventional cytotoxic drugs.

TARGETING MYC FOR CANCER THERAPY

Due to the great variety of genes and cellular responses under the control of Myc, targeting Myc itself will eventually influence many downstream regulatory pathways, crucial in both the induction and progression of cancer. Wang *et al.* recently showed that a sustain expression of c-Myc is required in a large number of tumor cell lines to maintain proliferation and that c-Myc depletion negatively affects cell survival by inducing arrest of the cell cycle at various stages [90]. Further evidence supporting the potential benefits of targeting Myc came from the work of Felsher *et al.*, who used a tetracycline-regulated system to modulate Myc levels in hematopoietic cells of transgenic mice [65]. Suppression of Myc induced tumor regression associated with a rapid proliferative arrest, differentiation and apoptosis of tumor cells, therefore indicating that the functional rescue of even a single genetic lesion may be sufficient to reverse tumorigenesis. Nevertheless, Myc can also activate apoptosis, a cellular response that would be rather preserved than lost in the context of therapeutic anti-tumor approaches [26, 91]. Jain *et al.* have addressed this issue using the same transgenic model mentioned above [65], where a fraction of animals developed osteogenic sarcomas expressing high levels of Myc and sharing some features with the human disease [92]. A brief inactivation of Myc was able to promote tumor regression and cellular differentiation into mature bone, while Myc re-expression resulted in activation of apoptosis instead of restoring cell proliferation [92]. This observation greatly supports the feasibility of Myc-targeting strategies, where a transient suppression of Myc may be sufficient to achieve a therapeutic effect.

Many attempts have been made so far to target Myc at different biological levels, as schematically described in Fig. (**1**). Down-regulation of Myc may be achieved at the transcriptional level, by either preventing the activation of the *myc* promoter [93] or destabilizing the *myc*-specific transcript by antisense strategies, which can be based on oligonucleotides [94] or on the more stable peptide nucleic acids [95]. Although they are rather susceptible to degradation by nucleases, small interference RNA (siRNA) molecules gave remarkable results in different tumor models, both *in vitro* and *in vivo*. By this approach, c-Myc depletion led to a decrease in cell proliferation and to inhibition of colony formation in soft agar assays, as well as a reduction in tumor

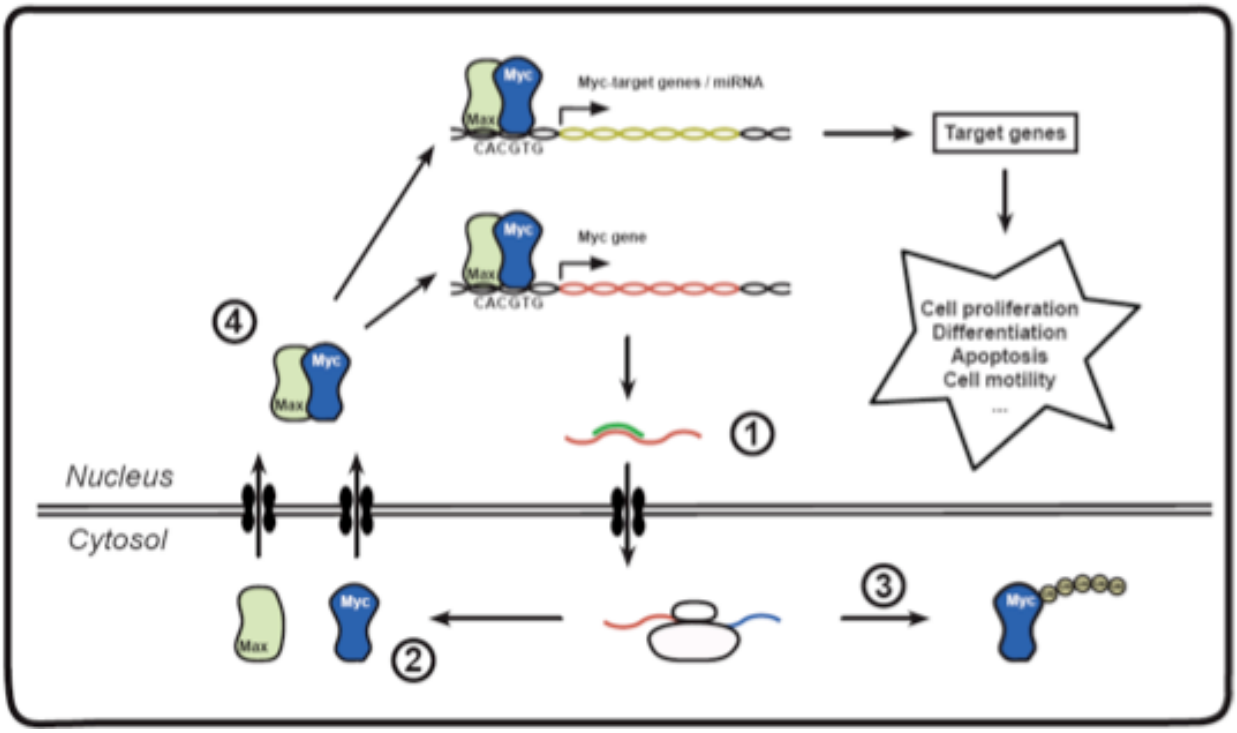

Fig. (1). Myc targeting at different levels. Either at the genetic level or at the protein level, Myc can be a suitable target to design new therapeutic strategies, aiming at eventually modulating Myc-dependent cellular events involved in tumorigenesis. Small interference RNA molecules, DNA-based oligonucleotides, and peptide nucleic acids represent diverse forms to deliver sequence-specific oligonucleotides, preventing translation or promoting degradation of *myc*-mRNA in the nucleus (1). In the cytosol, the protein can be destabilized or blocked by small-molecule inhibitors of different kind, usually chemically synthesized derivatives of natural toxic compounds (2). The stability of Myc can be also altered *via* indirect mechanisms, like by promoting the ubiquitin-dependent degradation of Myc (3). Moreover, the functionality of Myc can be affected by preventing the protein from interacting productively with the transcription partner Max, in the nucleus (4). Consequently, transcription not only of Myc target genes but also of *myc* itself, which is determined by auto-regulatory feedback mechanisms, is impaired. In turn, the downstream cellular responses normally elicited (e.g. cell proliferation, differentiation, and apoptosis) can be very significantly altered.

growth in nude mice [96]. Moreover, the silencing of Myc represents an experimental approach that is instrumental to the identification of new Myc target genes [97].

Interfering with Myc stability at the protein level, by stimulating the cytosolic proteasome-mediated degradation machinery, may represent a further strategy of intervention (Fig. (**1**)). A contribution in this direction has been provided by the identification of the ubiquitin-specific protease USP28, which stabilizes Myc in proliferating tumor cells [98]. This enzyme and other members of Myc-stabilizing complexes might be targeted in order to ultimately promote a more rapid turnover of the active form of Myc. Finally also, preventing the association of Myc to its partner Max would in turn affect the Myc-dependent regulation of transcription [99]. Antagonists of the Myc/Max dimerization represent a novel category of small-molecule Myc-specific inhibitors that are currently under investigation [100, 101].

Because of the multiple downstream signaling pathways directly or indirectly regulated by Myc, it is clear that targeting this oncogene represents a very powerful but, at the same time, a very complex strategy for therapeutic purposes. As mentioned already, Myc exhibits a dual function by promoting both proliferation and apoptosis [26], also implying that apoptosis can be prevented, eventually, upon Myc inhibition. This aspect should always be taken into consideration during optimization of therapeutic protocols, in particular when different approaches are combined, such as Myc-inhibitory agents and chemotherapeutic drugs. In fact, some anti-tumor compounds (e.g. cisplatin) can induce apoptosis in a way dependent on Myc expression level [102], and tumor cells may become therefore drug resistant upon Myc down-regulation [103].

A better elucidation of the Myc-dependent mechanisms inducing programmed cell death (i.e. identification of apoptosis-related Myc target genes) will help developing more efficient clinical strategies. Concomitantly, other therapeutic targets (downstream of Myc) should be also considered, in order to selectively activate Myc-dependent pathways not influencing apoptosis or, alternatively, to functionally reactivate pro-apoptotic signals. One relevant apoptotic pathway involves, for instance, the transcription factor MIZ-1 and the anti-apoptotic protein Bcl-2, whose encoding genes are both repressed by Myc [104]. A direct targeting of either of these genes would therefore rescue the pro-apoptotic functions of Myc also in a setup of Myc down-regulation [105].

Some of the strategies mentioned here have been investigated by *in vitro* and *in vivo* models of embryonal tumors, aiming at reducing the transforming potential of Myc. In the

Table 1. Myc Expression and Clinical Outcome

	Myc status	Clinical outcome
Neuroblastoma	Amplification of *MYCN*	Poor prognosis [92-94] Rapid tumor progression [92-94]
	High MYCN mRNA expression without gene amplification	Uncertain prognostic significance [95, 96]
Medulloblastoma	Amplification of *c-myc*	Poor prognosis, anaplasia [144, 154, 164]
	High c-Myc mRNA expression	Poor prognosis [147, 158]
	Low c-Myc mRNA expression	Favorable-risk prognosis [147, 158]

The change in expression of *MYCN* and *c-myc* are reported here in relation to the prognostic value, as emerged from the clinical data of neuroblastoma and medulloblastoma patients.

next sections, we will review the existing data on the involvement of Myc in the development of embryonal tumors of the central and peripheral nervous system, and on the main experimental studies currently on-going to selectively regulate Myc expression and function in these tumors. The major work so far has been done in NB, whereas only a few examples of translational research are available in the field of MB and AT/RT.

Myc IN NEUROBLASTOMA

Neuroblastoma (NB) is a tumor derived from primitive cells of the sympathetic nervous system and is the most common extra-cranial solid tumor in childhood [106]. The clinical outcome of NB can range from complete regression (mainly in infants) to rapid tumor progression and metastasis with poor prognosis [107]. Identification of the most common genomic alterations associated with the disease has allowed the classification of NB into low-, intermediate- and high-risk groups [108, 109]. Unfavorable tumors are characterized by deletions of 1p or 11q, unbalanced gain of 17q and/or amplification of *MYCN* [110]. Amplified *MYCN* has been proposed as an example of the first clinical application of cellular oncogenes, and is now established as a powerful molecular parameter to determine patient prognosis, as well as a basis for patient stratification [109, 111, 112] (Table **1**).

MYCN is localized at 2p23-24 [113] and it is found in double minutes (DM) and homogeneously staining chromosomal regions (HSR) when amplified [114]. *MYCN* can be amplified up to 50- or 100-fold, although duplications at 2p24 were also found in NB, maybe as precursors of further amplification [115]. Amplified *MYCN* was shown to be sensitive to differentiation-inducing signals (e.g. retinoic acid) that evoke a drastic decrease in the mRNA level of *MYCN* in NB cells [116]. This observation is in line with previous findings in undifferentiated neuroblasts that differentiate into ganglion cells by losing expression of *MYCN* [117].

The development of an effective therapy for NB has proved to be one of the major frustrations in pediatric oncology [118]. Over time, the favorable outcome of patients with stage I and II disease was recognized and such children received less treatment [118]. Those with stage III and IV were treated with increasingly aggressive regimens. The survival time improved a little, but not the survival rate. Until recently, the survival of stage IV-patients over one year of age has remained only about 10%. The development of more effective and also less toxic therapeutic schedules is, therefore, of great importance. An overview of some of the experimental approaches aimed at selectively interfering with Myc or Myc-dependent pathways in NB will be given here (Table **2**). Each study has put forward new interesting findings of therapeutic relevance that have the potential to be further developed into clinical approaches.

MYCN expression was silenced by using siRNA oligonucleotides, alone or cloned into expression vectors, with the result of inducing differentiation and apoptosis in NB cells [119]. A decrease in *MYCN* mRNA level was also achieved upon treatment with retinoic acid, which concomitantly induced neuronal differentiation and a reduction in cell proliferation due to cell cycle arrest in G1 [120]. Synthetic deriva-

Table 2. Myc Targeting

	Level	Strategy	Effect
Neuroblastoma	mRNA	Small interfering RNA [103]	Differentiation, apoptosis
		DNA antisense oligos [106]	Differentiation, decrease in cell proliferation
		Peptide nucleic acids [107, 108]	Decrease in cell proliferation, cell cycle (G1) arrest
		Retinoic acid/derivatives [104, 105]	Cell growth and cell cycle (G1) arrest, differentiation
	Protein	CR-UK compound collection [111]	Inhibition of Myc transcriptional activity
Medulloblastoma	mRNA	Small interfering RNA [167]	Cell proliferation and cell cycle (S) arrest
	Protein	Quassinoid derivatives (NBT-272) [169]	Cell proliferation, cell cycle (G1) arrest and apoptosis
		Resveratrol [167]	Cell proliferation arrest, apoptosis

The experimental strategies aiming at downregulating Myc in neuroblastoma and medulloblastoma are here summarized, divided into approaches targeting *MYCN*/*c-myc* at the mRNA or protein level.

tives of retinoic acid have also been screened for anti-proliferative potential in NB. Ponthan *et al*. reported about one particular derivative inducing apoptosis in SK-N-BE(2) and SH-SY5Y cells [121]. In addition, *MYCN*-specific antisense DNA oligonucleotides have been successfully tested for their capacity to decrease the proliferation rate and to induce cellular differentiation in the LAN-5 cell line [122]. The same antisense oligonucleotides were validated in a transgenic mouse model of human *MYCN*-induced NB, in which tumor incidence and progression could be decreased [122]. Another strategy employed the more stable peptide nucleic acids to target *MYCN* in NB cells with Myc overexpression. These molecules were used alone [123] or in combination with a somatostatin analogue for a better cellular uptake in the IMR32 cell line, thus eliciting an inhibition of cell proliferation and/or arrest of the cell cycle in phase G1 [124].

At a further biosynthetic level, proteins can also be targeted, by affecting for instance protein stability and ultimately preventing their function, such as the Myc transcriptional activity on its downstream targets. Recently, it has been shown that the stability of MYCN depends on Akt phosphorylation and inhibition of glycogen synthase kinase-3β (GSK3-β) [125]. Inhibition of phosphoinositide 3-kinase (PI3K), upstream of Akt, led to a rapid decrease in MYCN protein levels, resulting in inhibition of tumor growth [125]. Inhibitors of PI3K, of Akt, and of GSK3β have been evaluated for the development of clinical treatments of tumors bearing *MYCN* amplification [126]. Although possible side effects of inhibitors of the PI3K/Akt pathway include diabetes, targeting specific PI3K isoforms was shown to be a feasible approach *in vivo* [127, 128]. Another class of inhibitors was designed to block the activity of MYCN. As an example, Lu *et al*. reported on a library of chemical compounds, screened by using a cell-based reporter gene assay [129]. Eight substances (out of 2800) induced a reduction in luciferase activity greater than 50% and were selected for further investigation.

Sarkar and Nuchtern evaluated the potential of MYCN in cell-mediated immunotherapy [130]. They designed a MYCN-derived peptide containing a binding motif for the HLA-A1 allele of the Major Histocompatibility Complex-class I. Cytotoxic T lymphocytes responsive to this specific MYCN-epitope were able to selectively lyse NB cells from a HLA-A1-positive patient [130], suggesting that vaccine-based strategies could represent one possible approach to treat *MYCN*-amplified NB, provided that further experimental evidences will be given.

As an alternative to target MYCN itself, small-molecule inhibitors could be used against effector molecules downstream/upstream of Myc in Myc-related signaling pathways. The nucleoside analogue transcriptional inhibitor ARC has been recently identified and proposed as a pro-apoptotic compound, acting *via* a mechanism that involves the protein kinase Akt and triggers MYCN down-regulation [131]. Other signaling pathways have been also considered as therapeutic targets, such as the wingless (Wnt)/β-catenin pathway. Its alteration in the pathological onset is responsible, at least in part, for deregulating Myc and altering the expression profile of other oncogenes in high-risk NB with no *MYCN* amplification [132].

MDM2, a direct transcriptional target of MYCN and activated in NB [133], has been reported as a necessary and sufficient mediator of MYCN-dependent centrosome amplification by exerting a central inhibitory effect on p53 [134]. To functionally inactivate MDM2, the small-molecule inhibitor of the MDM2-p53 interaction nutlin-3 [135] was used. Nutlin-3, in fact, has shown a therapeutic potential for treatment of NB with *wild type* p53, by selectively activating p53-dependent apoptosis and inducing a block of the cell cycle in NB cells, regardless of the activation status of *MYCN* [136].

Not only conventional protein-coding genes may have an oncogenic function in NB, as well as in cancer development in general. MicroRNAs (miRNA) are non-translated RNA sequences that map to regions of genomic imbalance and can regulate the gene expression at a post-transcriptional level [137]. These molecules can either degrade or prevent the translation of a target mRNA, with consequences on gene expression. So far, two independent studies reported the differential expression of miRNA in primary NB samples with favorable and unfavorable clinical outcome [138, 139]. Although the predictive clinical value of miRNA expression was difficult to address, four miRNA sequences were identified in both studies to positively correlate with *MYCN* amplification. Recently, Schulte *et al*. [140] reviewed the involvement of miRNA in the pathogenesis of NB, underlining the relevance of two miRNA clusters in particular, which have been identified as tumor suppressor (miR-34a) and as oncogene (miR-17-92), respectively. Whereas the miR-17-92 cluster is a direct target of MYCN [139], the *MYCN* gene was found to be a target of miR-34a that induces cell cycle arrest and apoptosis [141, 142]. Even though investigating the role of miRNA in tumorigenesis is a relatively new field, the possibility to manipulate miRNA-mediated functions already represents a promising approach of anti-tumor gene therapy [137]. Inhibitors of miRNA have been administered *in vivo* [143, 144], and other chemically modified nucleic acids are predicted to be useful in the future to functionally replace tumor suppressor miRNA that are down-regulated in cancer.

In summary, different approaches can be suggested as potential novel therapies for the treatment of NB. In particular, we have mentioned strategies that aim at targeting the oncogene MYCN, which is overexpressed in many NB tumors with poor clinical outcome (Table **1**). So far, MYCN has been targeted at both the mRNA and protein levels in preclinical studies (mainly *in vitro*), which lead to very interesting preliminary findings and surely warrant further investigations *in vivo* (Table **2**).

Myc IN MEDULLOBLASTOMA

Medulloblastoma (MB) is the most common malignant tumor of the central nervous system in childhood and represents more than 20% of all pediatric brain tumors [145]. MB is characterized by aggressive clinical behavior and high-risk of leptomeningeal dissemination. Most metastatic and recurrent MB tumors are resistant to current therapeutic approaches, including high-dose chemotherapy with autologous hematopoietic stem cell rescue [146-148]. It is therefore of particular relevance to achieve a better understanding

of the molecular basis of MB pathogenesis, also in the view of discovering new therapeutic targets.

A better knowledge of the biology of MB has allowed the identification of some crucial signaling pathways involved in development and progression of MB. Sonic hedgehog (Shh) is a mitogenic glycoprotein secreted by cerebellar Purkinje cells, which provides a signal for the granule cell progenitor (GCP, the most abundant neuron in the cerebellum) to initiate cell proliferation [149]. The Shh and the Wnt signaling pathways [150-152] were found to be implicated in MB development [153-157], by inducing an upregulation of *MYCN* and consequently an increase of the cell proliferation rate [158-162]. High levels of MYCN in turn activate the cyclin D family (cell cycle-activators) and repress the expression of some cyclin-dependent kinase inhibitors eliciting the opposite effect (cell cycle-inhibitors) [163]. Increased expression and stability of MYCN have a synergistic effect, together with the activation of the Shh signaling pathway, in the induction of MB [164, 165].

Although the involvement of MYCN has come historically together with the first evidences of deregulation of signaling pathways in MB, another gene of the Myc family, c-Myc, has been found to be very relevant in the development of this pathology [166-176]. Both *MYCN* and *c-Myc* are amplified in 5 to 10% of MB patients [169, 177-180], with this rate increasing to 17% in the clinical subgroup of high-risk patients [171, 181]. Amplification of the *c-myc* gene and high level of the c-Myc gene product have both been used as clinical indicators of poor prognosis [173, 174, 182, 183] (Table **1**). Another complementary histopathological prognostic factor is the large cell/anaplastic (LC/A) phenotype, indicative of a very aggressive clinical behavior and outcome [169, 175, 180, 181, 184-189]. In contrast, low expression of c-Myc, alone or in combination with high TrkC-mRNA levels, has been proved to be strong predictors of a favorable outcome in MB [174, 190, 191].

Contrary to the substantial amount of preclinical studies in NB, the investigation of Myc-specific therapeutic approaches in MB is still in its infancy (Table **2**). To date, there are only two *in vitro* studies published. Zhang *et al*. used an antisense approach to silence c-Myc [192], which led to an inhibition of cell proliferation and to an arrest of the cell cycle in the S phase. In the same report, it was also suggested that Myc down-regulation is likely to represent the critical intermediate event responsible for the anti-proliferative effect of resveratrol in MB cells [193]. Another compound, the synthetic quassinoid derivative NBT-272, is currently under investigation in our group, on the basis of previous findings obtained in a panel of MB-derived cell lines [194]. In this report, NBT-272 was able to reduce cell proliferation and to block cell cycle progression. Although still preliminary, these studies may open the way to the use of small-molecule inhibitors in the treatment of Myc-overexpressing MB tumors.

Myc IN RHABDOID TUMORS

Rhabdoid tumors are rare but highly aggressive tumors that typically occur in infancy or early childhood [195, 196]. Rhabdoid tumors predominantly arise in the kidney and brain (atypical teratoid/rhabdoid tumors, AT/RT), but they can also be found in a deep axial location, such as the neck or the paraspinal region [197]. Different forms of rhabdoid tumors can be similar in their aggressiveness, histological features, and loss of function of *INI1/hSNF5* mapping on chromosome 22 [198, 199]. From the clinical experience, infants and children with rhabdoid tumors respond very poorly to chemotherapy and radiotherapy [200-202], although this remarkable resistance to both cytostatic drugs and radiotherapy has found no convincing explanation at the molecular level, to date.

INI1/hSNF5, initially identified as a host protein interacting with the human immunodeficiency virus type 1-integrase [203, 204], contributes to the functional core of the ATP-dependent chromatin-remodeling SWI/SNF complex [205]. The components of this complex have been shown to play a role in both activation and repression of gene transcription. In particular, *INI1/hSNF5* has been described as a tumor suppressor and its loss results in various aggressive cancers [206]. Point mutations or deletion of both copies of *INI1/hSNF5* are observed in approximately 70% of primary AT/RT, whereas an additional 20-25% of tumors have a reduced expression of INI1/hSNF5 at either the mRNA or protein level [207].

Ectopic expression of INI1/hSNF5 leads to cell cycle arrest in G1 and consequently prevents cell entry into phase S. This event is associated to down-regulation of a subset of E2F target genes, including cyclin A, E2F1 and CDC6 [208]. Notably, some genes of the E2F and cyclin family have also been identified as c-Myc targets [209]. Similarly to the other embryonal tumor entities discussed above, c-Myc is often up-regulated in rhabdoid tumors [210-213], even though no solid evidence of correlation with clinical parameters has been defined at present.

By using the yeast two-hybrid system approach [49, 214], c-Myc was identified as a component of the SWI/SNF macromolecular complex and as an interacting partner of INI1 [206]. The DNA-binding B-HLH-LZ motif at the COOH-terminus of Myc is responsible for recognition and binding to the conserved repeat-1 region of INI1. This interaction seems to have not only a structural, but also a functional relevance [206], by mediating the recruitment of the chromatin-remodeling complex SWI/SNF to the E-box of genes that are repressed upon chromatin condensation [215, 216]. The same SWI/SNF complex, however, can also activate transcription of c-Myc target genes that are involved in apoptosis [206, 217]. This evidence would therefore support the existence of a common SWI/SNF-dependent mechanism of chromatin remodeling, able to regulate both repression of pro-survival genes and activation of pro-apoptotic genes, eventually eliciting a tumor-suppression signal. Either effects on gene expression are probably dictated by a distinct combination of transcription elements of the Myc family [26, 38-40].

To investigate the mechanism of INI1/hSNF5-dependent tumor-suppression, Zhang *et al*. reintroduced the gene in the MON cell line carrying biallelic deletions at the *INI1/hSNF5* locus. As a consequence, cells were prevented from entering the S phase of the cell cycle [218], cell proliferation was inhibited, cell morphology changed, and the expression of cyclin D1 was repressed [219]. Because of the critical role of

cyclin D1 in cell proliferation [220-222], it is not surprising that cyclin D1 and other cell cycle-regulators are also often overexpressed in rhabdoid tumors [223].

Chromatin immunoprecipitation studies showed that cyclin D1 is a direct target of INI1/hSNF5, as well as of c-Myc [30]. In fact, cyclin D1 belongs to the group of genes, whose expression can be either activated or repressed, depending on the chromatin status and on the transcription factors that are engaged. Whereas Myc/Max dimers promote transcription of cyclin D1 [224], Myc can silence the same gene by a mechanism independent of Max, which does not interact with INI1 [199].

Taken together, these findings also suggest possible new targets along the Myc-dependent cell proliferation pathways, alternative to target Myc itself. Repression of cyclin D1 represents one attractive approach [225]. Possible ways of intervention are treatment with retinoic acid or siRNA molecules specific for cyclin D1, which are under investigation in other tumor models [226]. Furthermore, gene therapy aiming at reintroducing INI1/hSNF5, which is able to arrest cell growth in AT/RT, could also be considered in order to restore the defective copies of this gene in patients.

CONCLUSIONS AND FUTURE PERSPECTIVES

We have summarized here the current progresses in the design of novel therapeutic strategies for the treatment of embryonal tumors based on targeting Myc, with a particular emphasis on NB, MB, and AT/RT. Deregulation of the *myc* proto-oncogene is often linked to the development of a more aggressive disease and to poor prognosis in patients. Myc is a family of multifunctional transcription factors that, directly or indirectly, determine a multitude of cellular events responsible for tumorigenesis. Therefore, the possibility to target Myc or Myc target genes in order to eventually manipulate Myc-dependent signaling pathways, like those leading to cell proliferation and apoptosis, is particularly attractive. The Myc targeting approaches under investigation, although still at the preclinical stage, range from RNA/DNA-based oligonucleotides to small-molecule inhibitors. These strategies could complement the administration of more conventional drugs to patients, aiming at less toxic and more selective treatment schedules. Among all, the RNA interference (RNAi) approach seems to be particularly promising to silence disease-causing or disease-promoting genes [227, 228]. Although some aspects (i.e. drug delivery, possible immunoreactivity, off-target effects) have still to be better understood and optimized, the recent fast progress in the field has triggered some optimism about the development of RNAi-based therapeutic tools for translational research in the clinic [229]. Finally, the increased knowledge about different tumor entities similarly characterized by Myc deregulation, and the preliminary results obtained by employing RNAi or other approaches, will certainly contribute to make important steps forward in developing novel therapies for embryonal tumors.

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Quassinoids: From Traditional Drugs to New Cancer Therapeutics

G. Fiaschetti[1], M.A. Grotzer[1], T. Shalaby[1], D. Castelletti[1] and A. Arcaro*[,2]

[1]*Department of Oncology, University Children's Hospital, Zurich, Switzerland*

[2]*Department of Clinical Research, University of Bern, Switzerland*

Abstract: Quassinoids are a group of compounds extracted from plants of the *Simaroubaceae* family, which have been used for many years in folk medicine. These molecules gained notoriety after the initial discovery of the anti-leukemic activity of one member, bruceantin, in 1975. Currently over 150 quassinoids have been isolated and classified based on their chemical structures and biological properties investigated *in vitro* and *in vivo*. Many molecules display a wide range of inhibitory effects, including anti-inflammatory, anti-viral, anti-malarial and anti-proliferative effects on various tumor cell types. Although often the exact mechanism of action of the single agents remains unclear, some agents have been shown to affect protein synthesis in general, or specifically HIF-1α and MYC, membrane polarization and the apoptotic machinery. Considering that future research into chemical modifications is likely to generate more active and less toxic derivatives of natural quassinoids, this family represents a powerful source of promising small molecules targeting key pro-survival signaling pathways relevant for diverse pathologies. Here, we review available knowledge of functionality and possible applications of quassinoids and quassinoid derivatives, spanning traditional use to the potential impact on modern medicine as cancer therapeutics.

Keywords: Anticancer therapeutics, quassinoids, *Simaroubaceae*, tumor.

QUASSINOIDS AND ANTI-CANCER ACTIVITIES

Natural products have been a popular source of medicinal agents for thousands of years [1, 2]. More than one thousand species of plants possess significant antineoplastic properties, and over 50% of the currently used anti-cancer agents are derived from natural sources [3]. We review what is known about the quassinoid family here. A class of nortriterpenoids isolated exclusively from various species of the Simaroubaceae plant family, quassinoids possess various pharmacological properties including antitumor, antiviral, antimalarial, anti-ulcer, and anti-inflammatory activities [4-9]. Since the discovery of quassin and neoquassin in 1937, hundreds of quassinoids have been isolated and classified [4]. Several novel quassinoids with antitumor properties have been described in the last decade, and new insights into the mechanisms of action are coming to light.

Most quassinoids can be categorized into 5 groups, based on the carbon backbone (C18, C19, C20, C22 and C25 types) [10, 11]. (For detailed descriptions of structural characteristics, chemical features, and biogenesis of quassinoids see [4, 10]). Many quassinoids display a wide range of biological properties *in vitro* and *in vivo*, and structure-activity relation (SAR) studies have been performed to better clarify their bio-activities [4]. Here we focus on research investigating quassinoids possessing antitumoral properties conducted in the last decade. These efforts have isolated new compounds, conducted SAR studies and advanced understanding of biological mechanisms behind anti-cancer activity.

That quassinoids are a class of inhibitor for eukaryotic protein synthesis is well supported [12-14], although how the distinct compounds exert this effect is still not fully understood. The ability to interfere at the peptidyl-transferase site, thus preventing peptide bond formation and terminating chain elongation, has been proposed as the major mechanism responsible for antineoplastic activity [13]. Recent findings show that quassinoids are not just protein synthesis inhibitors, and that their anti-tumorigenic activity is likely associated with other properties. Biological effects including induction of apoptosis, down-regulation of the MYC oncogene, inhibition of elF4E phosphorylation, and alteration of membrane polarization have been described. It has also been reported that quassinoids regulate DNA and RNA synthesis, however, inhibition of nucleic acid synthesis may be a direct consequence of the blocked protein synthesis [15, 16]. A recent study investigated several quassinoids in a high-throughput screen for inhibitors of the AP-1 transcription factor, a known regulator of carcinogenesis [12]. The cytotoxic activity of these quassinoids did not correlate with only the inhibition of the AP-1 or NF-κB transcription factors, indicating that other mechanisms of action may play a role.

Generally, high cytotoxic activity is associated with molecules possessing 4-ring skeletons [10]. The α,β-unsaturated ketone and the α-hydroxyl group adjacent to the carbonyl group in the A-ring (see compound 2 for ring nomenclature) are structural features that correlate with a high potency against cancer. A hydroxyl at C-1 or C-3 (see compound **1** for carbons nomenclature) appears to be important for anti-cancer activity. Potent compounds often possess an intramolecular hydrogen bond between the hydroxyl and the oxygen of the A-ring enone, rendering the enone more susceptible to nucleophilic attack. Kupchan proposed this [17] as a plausible mechanism behind anti-tumoral activity. An ester side chain at C-15 is also associated with drug potency and spectrum of response [10]. Free hydroxyl groups and an oxygen-methylene bridge in the C-ring are also correlated with higher biological activity. Analogs lacking the D-ring lactone have been shown to be less active [18]. It is likely that the selectivity for a particular cellular target or pathway member are driven by the structural differences of quassinoids.

*Address correspondence to this author at the University of Bern, Department of Clinical Research, Tiefenaustrasse 120c, CH-3004 Bern, Switzerland; Tel: ++41 31 308 8029; Fax ++41 31 308 8028;
E-mail alexandre.arcaro@dkf.unibe.ch

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NEW INSIGHTS ABOUT TRADITIONAL DRUGS

Bruceantin (**1**) was first isolated from Brucea antidysenterica, a tree traditionally used in Ethiopia and Asia to treat cancer [19]. After the discovery of its potent anti-leukemic activity [17], several studies were performed to investigate the biological properties of bruceantin and the entire class of quassinoids. A new interest in bruceantin has arisen in the past decade, and bruceantin is currently the most investigated quassinoid [5, 15, 20]. It has been evaluated in three separate phase I clinical trials in patients with various types of solid tumors, and in two separate phase II trials for adult patients with metastatic breast cancer [21] and malignant melanoma [22]. These clinical trials failed due to toxicity (hypotension, nausea, vomiting and fever) and poor efficacy [12, 21].

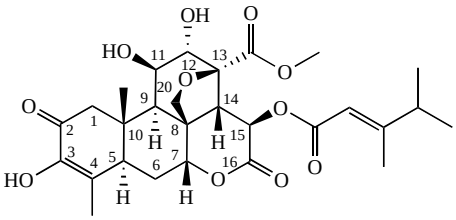
Cuendet *et al*. identified new activities of bruceantin in 2004, while investigating its effects on a number of leukemia, lymphoma and myeloma cell lines [16]. Bruceantin induced apoptosis in leukemia cells (HL-60 and RPMI 8226) *via* the proteolytic processing of procaspases-3, -8, and -9, and the cleavage of the caspase-3 substrate, poly-(ADP-ribose)-polymerase (PARP). Caspase-mediated proteolytic processing of the BH3-interacting domain death agonist (BID), a pro-apoptotic member of the Bcl-2 family responsible for amplification of the caspase cascade [23], was also observed. The activation of both caspase-mediated and mitochondrial apoptotic pathways was confirmed by the observation of disrupted mitochondrial membrane potential triggered by some quassinoids [24]. An *in vivo* multiple myeloma model (RPMI 8226 human xenografts in SCID mice) confirmed that apoptosis was significantly elevated in tumors derived from animals treated with bruceantin (2.5-5 mg/kg). Bruceantin treatment also led to a significant regression of these tumors [16].

Bruceantin may also be a potential new drug for treatment of malignances characterized by over-expression of the MYC oncogene [16, 25]. The over-expression of this oncogene is associated with tumor aggressiveness and poor cellular differentiation [26]. MYC was down-regulated in both *in vitro* and *in vivo* cancer models upon bruceantin treatment, suggesting a key role for MYC in the mechanism of bruceantin-mediated cell death and cell differentiation. Many other quassinoids possess the ability to inhibit this oncogene. A mechanism of action involving the down-regulation of c-MYC was associated with the brusatol-mediated induction of leukemic cell differentiation and observed cell cycle arrest in the G1 phase [27]. RNA transcripts for c-MYC were reduced to a lesser extent than the protein, suggesting that quassinoids regulate c-MYC expression at the translational and/or post-translational levels.

Recently brucein-D (**2**), another member of the quassinoid family, has been extensively investigated [5, 28]. This compound is extracted from Brucea javanica, a shrub traditionally used in Asia to treat malaria and cancer [29]. Brucein-D differs from bruceantin by its lack of ester groups in positions 13 and 15. This molecule was shown to be the major chemical constituent of the ethanolic extract of Brucea javanica fruit in 2008, and it was shown to be responsible for the inhibition of cell proliferation and induction of apoptosis observed in a model of human pancreatic adenocarcinoma [5, 28]. Brucein-D also decreased the growth of human pancreatic tumors grown as xenografts in nude mice. *In vitro*, Brucein-D inhibited the growth of three pancreatic cancer cell lines (PANC-1, SW1990 and CAPAN-1), and exerted a low cytotoxicity in non-tumorigenic human Hs68 fibroblasts. Treated cells showed typical apoptotic morphology characterized by chromatin condensation together with an increased amount of cleaved DNA/histone complexes. Similarly to bruceantin, exposure to brucein-D induced proteolytic activation of procaspases-3, -8 and -9. In addition, treatment attenuated expression of the anti-apoptotic protein, Bcl-2, while augmenting the expression of the pro-apoptotic protein, Bak. In 2010, Lau *et al*. more deeply delineated the mechanisms associated with brucein-D-mediated apoptosis in pancreatic cancer cells [30]. Brucein-D is capable of depleting intracellular levels of glutathione, an antioxidant which protects cells from reactive oxygen species, resulting in increased generation of superoxide-free radicals. The oxidative stress, in turn, provokes the activation of the p38-mitogen-activated protein kinase (p38 MAPK). The MAPK cascade regulates a variety of cellular responses to stress, inflammation and other signals, and its activation is often necessary for cancer cell death induced by several chemotherapeutics [31-33]. The ability of brucein-D to activate the p38-MAPK-regulated pro-apoptotic pathway underlines the potential of quassinoids as cell death inducers.

Brucein-D was also found capable of altering another well known tumorigenesis-related pathway. The nuclear factor kappa B (NF-κB) family of transcription factors is implicated in regulation of cell proliferation, apoptosis, invasion and angiogenesis in a variety of tumor malignancies [32]. Many studies revealed an important role of NF-κB in modulating cancer cell resistance to drugs and radiation therapy [34]. Moreover, it has been shown that inhibition of NF-κB can lead to tumor cell death and growth inhibition [35]. Impairment of this pathway has been suggested as a good strategy to treat certain cancer subtypes [36]. Brucein-D treatment inhibits NF-κB activity by augmenting the protein level of the IκBα inhibitory subunit, which sequesters NF-κB in the cytoplasm preventing its nuclear translocation [30]. A distinct mechanism of action involving NF-κB was described in a study on HL-60 leukemic cell differentiation induced by brusatol (**3**), a quassinoid extracted from brucea species [25, 27]. In 2003, Cuendet *et al*. [25] showed that brusatol up-regulates the mRNA levels of three members of the NF-κB family, NF-κB1 (p105/p50), NF-kB2 (p100/p52) and RelA (p65). Phosphorylation of the NF-κB inhibitor, IκBα, was also induced, resulting in enhanced translocation of NF-κB from the cytoplasm to the nucleus, transcriptional activation and the consequent induction of HL-60 cell differentiation.

Searching for novel plant-derived chemotherapeutics and chemopreventive agents, in 2001 Mata-Greenwood *et al*. [37] investigated the structure-activity relationships of a set of natural quassinoids (including brusatol and bruceantin) and semi-synthetic analogs of brusatol (**4-8**), glaucarubolone (**9-42**) and quassin (**43-47**). Most of the quassinoids showed activity as either cytotoxic or antiproliferative agents and/or as inducers of cellular differentiation. The parental compound, brusatol, was the most potent inducer of cellular differentiation, and inhibition of proliferation was primarily achieved by inducing G0/G1 arrest in the cell cycle. From a



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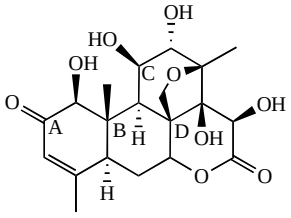

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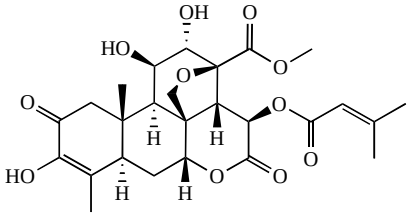

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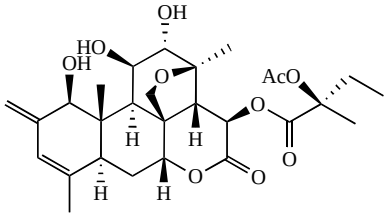

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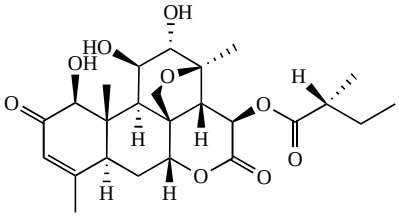

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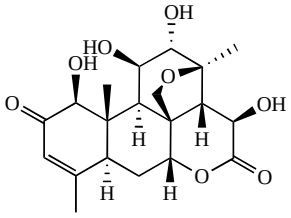

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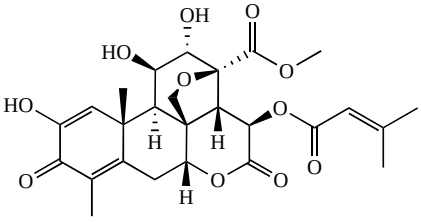

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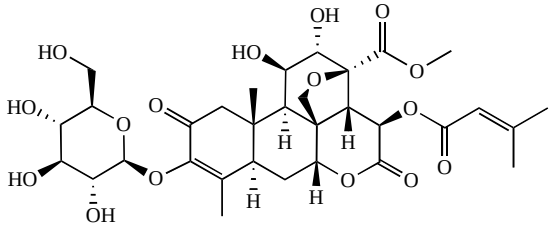

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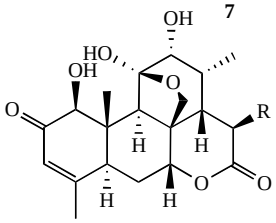

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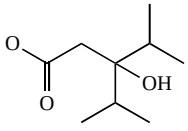

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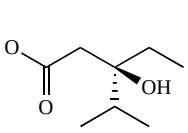

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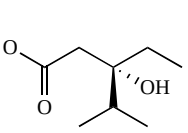

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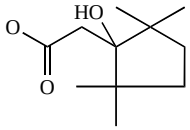

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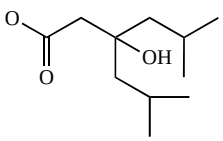

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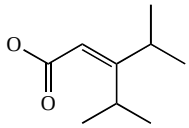

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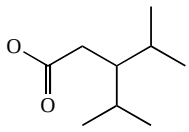

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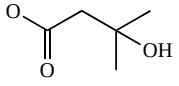

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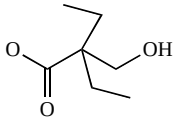

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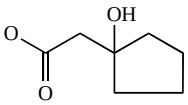

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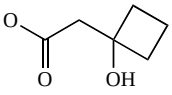

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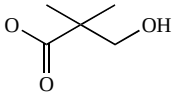

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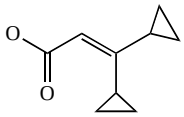

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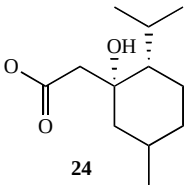

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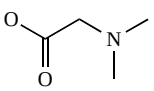

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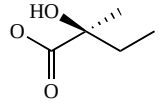

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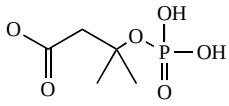

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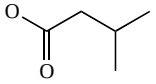

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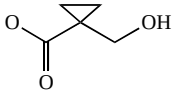

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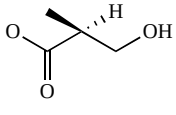

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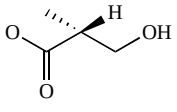

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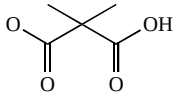

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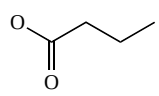

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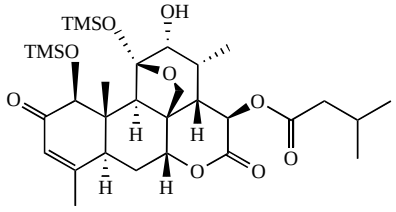
(Fig. 1). Contd…..



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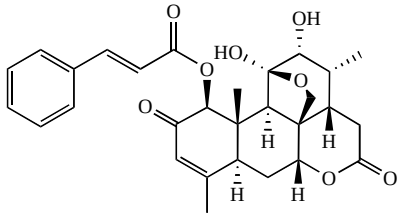

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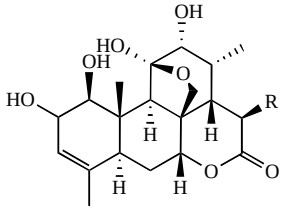

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39	OGlu
40	H
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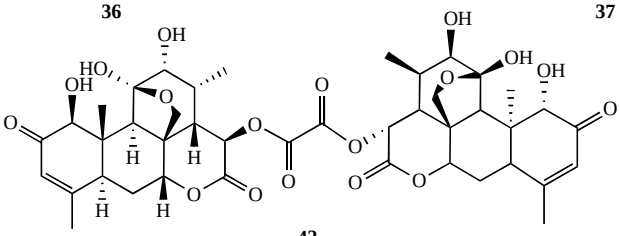

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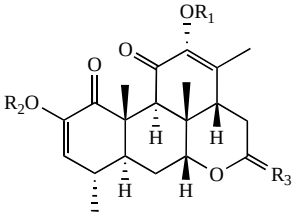
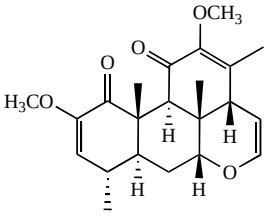


	R1	R2	R3
43	CH_3	CH_3	O
44	H	H	O
45	CH_3	CH_3	H/OH



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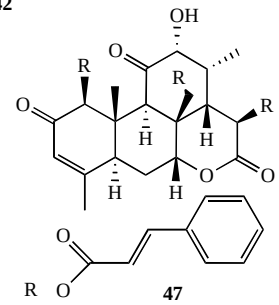

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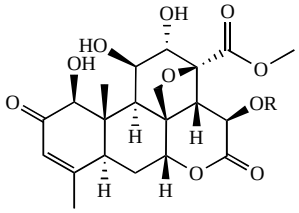
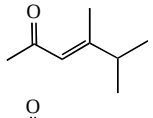
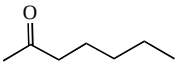


48	H	**49**	OAc	**50**	



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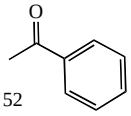

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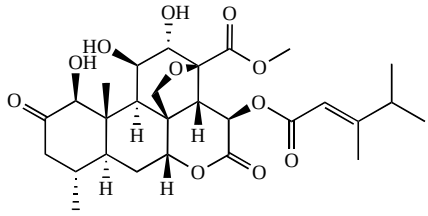

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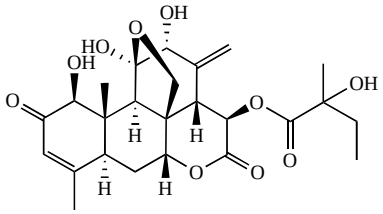

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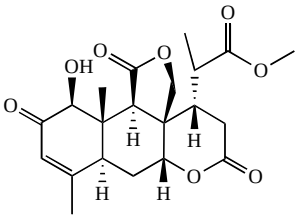

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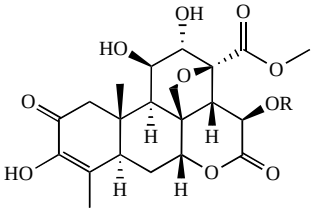
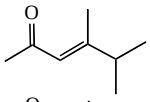
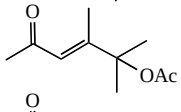
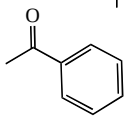
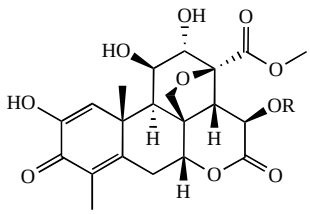
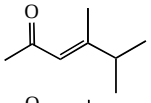
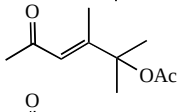
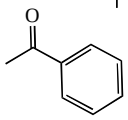
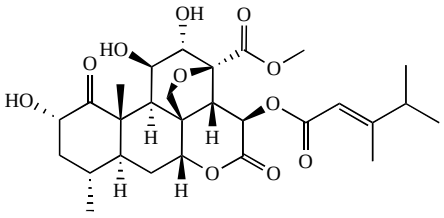


56	
57	
58	



59	
60	
61	



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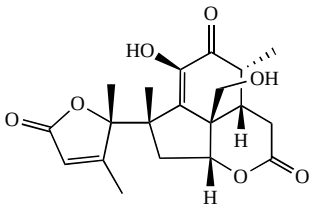

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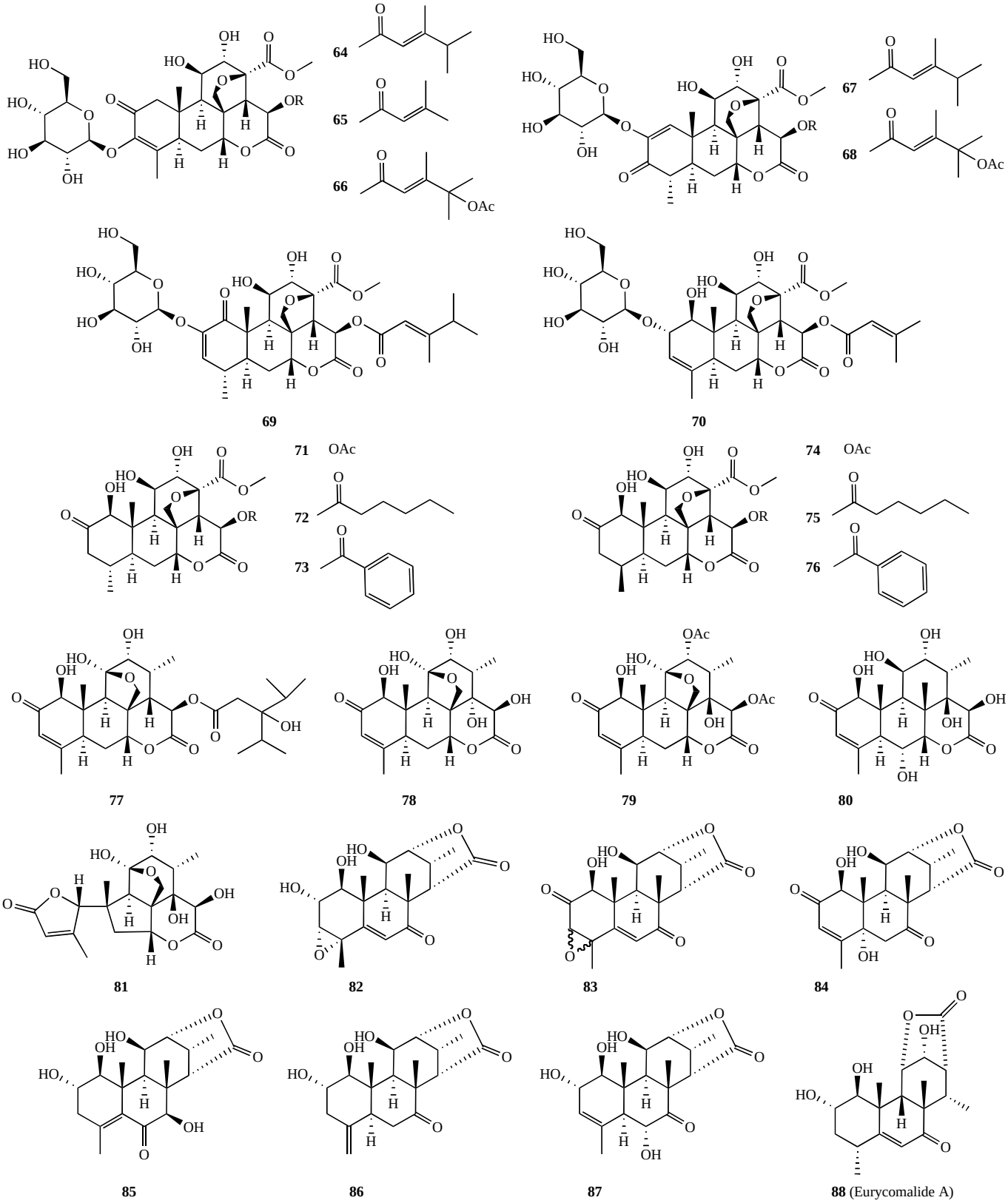
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71 OAc
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74 OAc
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88 (Eurycomalide A)

(Fig. 1). Contd…..

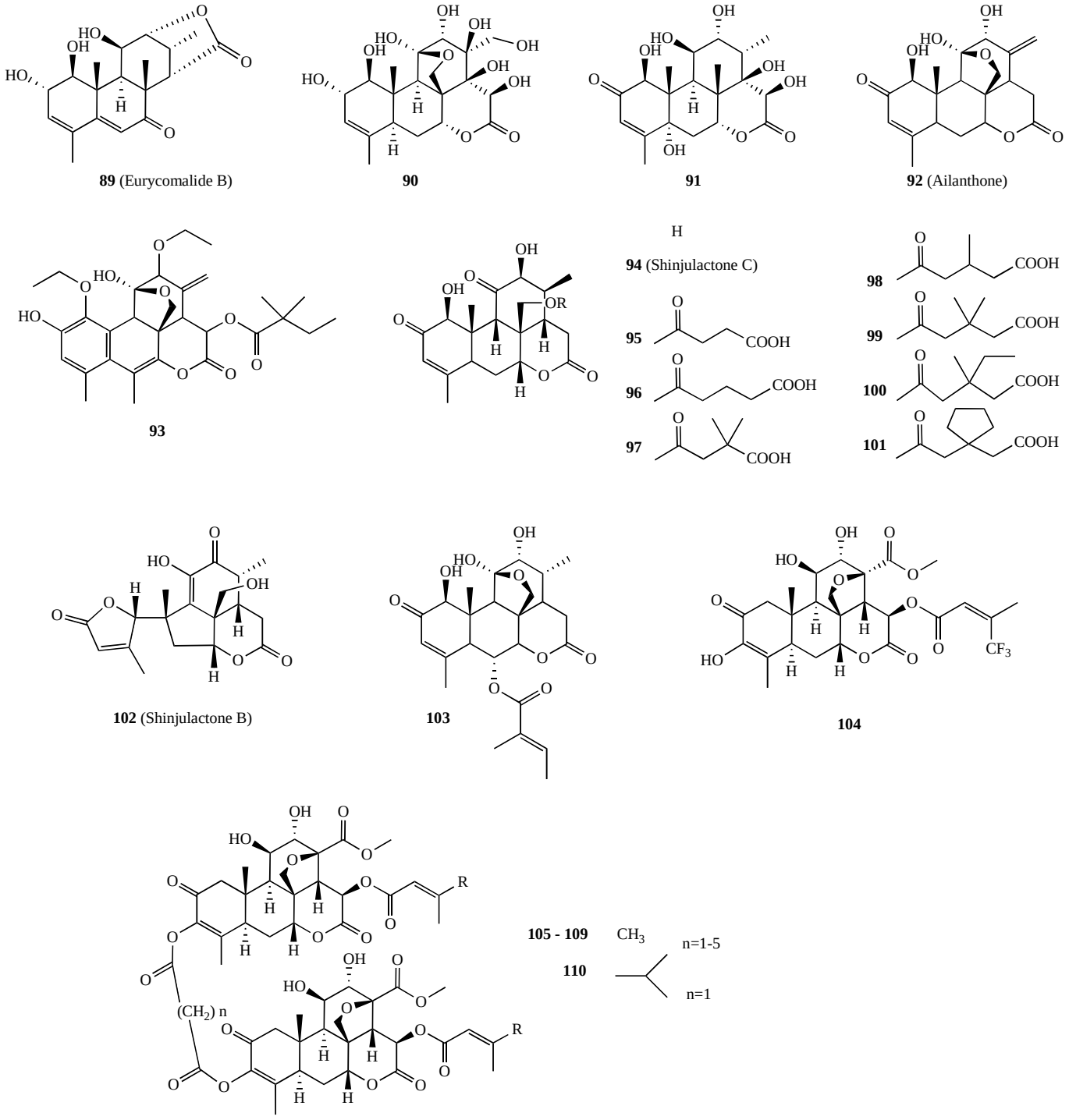

Fig. (1). Chemical structures of anticancer quassinoids described in this review.

structure-activity perspective, an hydroxylated ring at the β-position of the ester side chain at C-15 was correlated with the ability to induce cellular differentiation, while low potency and the ability to inhibit DNA synthesis were associated to the absence of a side chain at C-15 (**6**, **9**, and **27**). Quassinoids with low activity lacked either the epoxy-methano bridge in the D-ring (43-47) or a free hydroxyl group at positions 1, 3, 11 and 12 (i.e. due to glycosylation, **8**, **35**, and **39**). Based on the comparison of members with varied positioning of the epoxy-methano bridge, the direction of the bridge appeared not to be correlated with inhibition of cell growth or protein synthesis, but the lack of an ether bridge was associated with inactivity [12]. Even though this study showed no correlation between the lipophilicity of the ester side chain and the induction of HL-60 cell differentiation, the nature of the side chain appeared to be important. A branched side chain, resulting from the addition of alkyl groups (**13**, **18**), and the presence of hydroxyl substituents at

the α-position of the side chain (**10**, **17**) correlated with increased potency. Alkyl substituents in the α-position (**18**, **22**, **26**, **30**-**33**) have been associated with an increased selectivity for anti-proliferative activity versus cytotoxicity, but decreased potency to induce differentiation. Analogs possessing side chains with cyclic rings in the α-position (**13**, **19**, **20**, and **24**) showed increased selectivity for induction of differentiation and anti-proliferative/cytotoxic activity. A smaller set of quassinoids (**1**, **3**, **12**, **16**, **18**, **20**, **27**, and **34**) was investigated in the same study as inhibitors for the formation of pre-neoplastic lesions in the mouse mammary organ culture (MMOC) model [37]. Analogs possessing alkyl substituents at the α-position of the C-15 ester side chain (**1**, **3**, **18**, and **34**) showed high activities, in contrast to what was observed in HL-60, where potency was correlated with the presence of β-branched ester side chains.

The reduction in effectiveness of chemotherapeutic drugs caused by drug-resistance is one of the major problems in the fight against cancer [38]. Murakami *et al*. [39] examined **23** quassinoids (**48**-**76**) as inhibitors of three different multidrug-resistant human tumor cell lines. Compounds **49**-**54** and **56**-**58** were significantly cytotoxic against all three drug-resistant variants, with compounds **53** and **56** exhibiting the highest activity. The reduction of the C-3 double bond (**53**) increased activity, while the loss of the C-15 side chain (**48**) and/or the interchange of the position of the C-1 hydroxyl and C-2 carbonyl groups (**62**) markedly reduced potency. Moreover, the quassinoid glycosides (**64**-**70**) appeared to be less active than the corresponding aglycon quassinoids (**56**, **57**). In the same work, six hydrogenated derivatives (**71**-**76**) demonstrated significant inhibitory effects against 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced Epstein–Barr virus early antigen (EBV-EA) activation. All the derivatives showed slightly stronger inhibitory effects than the original compounds (**49**, **51**, and **52**), with compound 71 being the most potent. From the structure–activity relationship point of view, an α-oriented methyl group at C-4 resulted in stronger inhibitory effects than β-oriented groups. The reduction of the double bond in the A-ring led to stronger inhibition of EBV-EA activation.

NOVEL ANTI-TUMORAL QUASSINOIDS

Simaroubaceae plants have remained an interesting source of novel potential anti-neoplastic agents [4]. In 2004, the group of Kuo *et al*. [40] undertook the chemical investigation of the methanolic extract of the root of Eurycoma longifolia, a tree popular in Asia as a source of a wide spectrum of medications [41]. Along with 61 known compounds from several classes, four as yet undescribed quassinoids were isolated: two novel C19-skeleton quassinoids, eurycomalide A (**88**) and eurycomalide B (**89**); and two klaineanone-type C20 quassinoids, 13b,21-dihydroxyeurycomanol (**90**) and 5a,1-4b,1-5b-trihydroxyklaineanone (**91**). These compounds showed cytotoxic activity against human lung cancer (A549) and breast cancer (MCF-7) cell lines.

Miyake *et al*. [42] performed a detailed phytochemical investigation on Eurycoma longifolia in 2009. Twenty-four quassinoids were isolated, including ten new structurally diverse quassinoids. The new compounds included two eurycomanone-type C20 quassinoids (**78**, **79**), one klaineanone-type C20 quassinoid (80), one C19 quassinoid (**81**) with a 1,2-seco-1-nor-6(5f10)-abeo-picrasan-2,5-olide skeleton, and six eurycomalactone-type C19 quassinoids (**82**-**87**). For the first time, an α-oriented hydroxyl group at C-14 (**78**) and a 3,4-epoxy group (**82**, **83**) were reported in eurycomalactone-type quassinoids. The isolated compounds were tested for cytotoxic activity against the highly metastatic human fibrosarcoma cell line, HT-1080. Eurycomalactone-type quassinoids possessing an α,β-unsaturated ketone carbonyl at C-2 were found to be cytotoxic, confirming the α,β-unsaturated ketone as a chemical feature associated with high anti-proliferative activity.

Rosati *et al*. [43] isolated a novel quassinoid, Picrasa-3,13(21)-diene-2,16-dione-11,20-epoxy-1,11,12-trihydroxy,(1β,11 β,12α)-(9Cl) (ailanthone) (**92**) from Ailanthus excelsa, a plant traditionally used to treat cancer in Asian folk medicine [44]. Ailanthone induced the appearance of hypodiploid cells in 50% of Jurkat leukemia cell cultures. Apoptosis appeared to be induced by direct alteration of mitochondrial membrane permeability, induction of mitochondrial membrane depolarisation and activation of caspase-3.

Another novel quassinoid (AECHL-1) (**93**) was isolated from chloroform extract of Ailanthus excelsa by Lavhale *et al*. [45] in 2009. *In vitro*, AECHL-1 induced growth inhibition associated with a S/G2-M arrest in three different human tumor cell lines derived from breast carcinoma (MDA-MB-231), breast adenocarcinoma (MCF-7) and prostate carcinoma (PC3). AECHL-1 triggered G1 arrest in the mouse melanoma cell line, B16F10. The compound was tested *in vivo* on B16F10 allografts in C57BL/6 mice and on MCF-7 xenografts in nude mice. Injections of AECHL-1 into the tumor sites inhibited tumor growth in both models. Apoptosis was induced together with an increase in the phosphorylation level of p53. A reduction in the expression of the MYC oncogene and of two cell cycle regulatory proteins, cyclin-D1 and Cdk-4, was also observed. AECHL-1 also acts as a microtubule-damaging agent. Treated MCF-7 cells showed microtubule disruption, increased density of cellular microtubules and formation of thick microtubule bundles surrounding the nucleus.

Ailanthus altissima is another plant of the Simaroubaceae family, which has been traditionally used in Asia to treat cancer [46]. From this plant, Tamura *et al*. [7] isolated two new quassinoid molecules, shinjulactone-B (**102**), and shinjulactone-C (**94**). The latter possesses a unique skeleton among quassinoids, and the molecule has been used as parental compound to generate seven derivatives containing 20-O-acyl groups with terminal acid functionalities (**95**-**101**). To investigate their anti-tumor activities, these compounds were tested in a short-term *in vitro* assay of TPA-EBV-EA activation in Raji cells. All compounds showed inhibitory effects on EBV activation, and were only weakly cytotoxic for the Raji cells. From the structure–activity relationship point of view, the succinate derivatives (**95**, **97**) possessed higher inhibitory activities against EBV-EA activation than either the parental molecule or the glutarate derivatives (**96**, **98**–**101**). The strongest inhibitory effect was achieved by the 3',3'-dimethylsuccinate (**97**) derivate. It was also reported that the inhibitory activity was lessened if the substituted

Table 1. Antitumoral Quassinoids: Biological Effects and Model of Investigations

Quassinoid	Biological effects	Model	References
1 (Bruceantin)	↓ Protein Synthesis ↓ Proliferation ↑ Cell differentiation ↑ Apoptosis ↑ Caspase-3,-8-9 ↑ PARP; BID; Cytochrome c ↓ MYC ↓ Tumor (*in vivo*) ↓ pre-neoplastic lesion formation	Leukemia cells (HL-60) Lymphoma cells (RPMI 8226) Myeloma cells (U266 and H929) RPMI 8226 human-SCID xenograft Mouse mammary organ culture model	[17] [15] [16] [21] [22]
2 (Brucein-D)	↓ Protein Synthesis ↓ Proliferation ↑ Apoptosis ↑ Caspase-3,-8-9 ↑ Bcl-2 ↓ Bak ↓ Intracellular GSH ↑ NADPH oxidase ↑ p38-MAPK ↓ NF-kB (↑ IκBα) ↓ Tumor (*in vivo*)	Human pancreatic tumor cells (PANC-1, SW1990, CAPAN-1) Xenograft human pancreatic tumor in nude mice	[5] [30] [5]
3 (Brusatol)	↓ Protein Synthesis ↓ DNA Synthesis ↓ Proliferation ↑ Cell differentiation ↓ MYC ↑ NF-kB (↓ IκBα) ↓ pre-neoplastic lesion formation	Leukemia cells (HL-60) Mouse mammary organ culture model	[12] [27] [25]
4 - 47 (18, 34)	↓ DNA Synthesis ↓ Proliferation ↑ Cell differentiation ↓ pre-neoplastic lesion formation	Leukemia cells (HL-60) Mouse mammary organ culture model	[37]
48 – 76	↓ Proliferation	Human nasopharynx epidermoid carcinoma (KB) P-glycoprotein multidrug resistant variant (KB-VIN) MRP multidrug resistant cells (KB-7d and KB-CPT)	[39]
77 (NBT-272)	↓ Proliferation ↓ MYC G_1/ S arrest ↓ Protein translation ↓ p-elF4E ↓p-4EBP-1 ↓ AKT ↓ERK ↑ Autophagosome formation ↓ Tumor growth	Human embryonal tumor cell lines: Medulloblastoma (D341, D425, DAOY); Neuroblastoma (SK-N-BE, SH-SY5Y, WAC-2); Retinoblastoma (Y79, WERI); Ewing's sarcoma(A673, TC71); Malignant rhabdoid tumor (LP, MON)	[8] [59] [63]

(Table 1). Contd…..

Quassinoid	Biological effects	Model	References
78 - 87	↓ Proliferation	Highly metastatic fibrosarcoma cells (HT-1080)	[42]
88 - 91	↓ Proliferation	Lung cancer cells (A549) Breast cancer cells (MCF-7)	[40]
92 (Ailanthone)	↑ Hypodiploidy ↑ Apoptosis ↑ Caspase-3 Depolarization of mitochondrial membrane Alteration of membrane permeability	Leukemia cells (Jurkat)	[43]
93 (AECHL-1)	↓ Proliferation ↑ Apoptosis S/G_2-M arrest G_1 arrest (B16F10 cells) ↑ Apoptosis ↑ phospho-p53 ↓ MYC ↓ cyclin-D1 ↓ cdk4 Microtubule disruption ↓ Tumor (*in vivo*)	Mouse melanoma cells (B16F10) Human breast carcinoma cells (MDA-MB-231) Human breast adenocarcinoma cells (MCF-7) Human prostate cells (PC3) Human embryonic kidney cells (HEK 293) Nude mice xenograft with MCF-7 cells C57BL/6 mice allograft with B16F10 cells	[45]
94 - 102 (94 - Shinjulactone-C) (102 -Shinjulactone-B)	↓ Tumor promoting activity	TPA-induced EBV-EA in Raji cells	[7]
103 (6α-tigloyloxy-chaparrinone)	↓ HIF-1α ↓ VEGF ↓ erythropoietin ↓ MAPK ↓ MNK-1 ↓ elF4E	Human gastric adenocarcinoma cells (MKN-45 and AGS) Human hepatocellular carcinoma cells (Hep3B) Human breast cancer cells (MDA-MB-435)	[14]
104	↓ Proliferation	Panel of tumor cell lines	[58]
105 - 110	↓ Proliferation	Leukemia cells (P-388)	[57]

Symbols: [(↑ = Activation) (↓ = Inhibition)].

group of the glutarate became bulky, [3'-ethyl-3'-methylglutarete (**100**) and 3'-tetramethylene-glutarate (**101**)].

A novel mechanism of action among quassinoid compounds was described in 2008 by the group of Jin *et al*. [14]. They identified 6α-tigloyloxychaparrinone (compound **103**), a bruceantin analog carrying a tigloyloxy side chain in position 6. This quassinoid is able to interfere with different cellular mechanisms commonly altered in cancer [29, 47]. Compound **103** showed the particular ability to inhibit hypoxia-inducible factor-1α (HIF-1α), a central mediator of cellular responses to low oxygen. The effect was achieved by suppressing the phosphorylation of upstream elements of HIF-1α (ERK1/2, MNK1 and eIF4E) [14]. HIF-1α and its upstream elements represent attractive targets for future cancer therapeutics. For this reason, 6α-tigloyloxychaparrinone is a potentially interesting novel agent for future investigations [48, 49]. HIF-1α is over-expressed in certain human cancers, usually as a result of intratumoral hypoxia as well as genetic alterations, and it is crucial to many aspects of tumor biology, including angiogenesis, cell survival, glucose metabolism and invasion [50, 51]. Various human cancer cell lines were used for preclinical testing of the 6α-tigloyloxychaparrinone quassinoid. The compound dose-dependently decreased the hypoxia-induced accumulation of HIF-1α protein, and consequently, the expression of two HIF-1 target genes, vascular endothelial growth factor

(VEGF) and erythropoietin. HIF-1α inhibition was achieved without affecting the level of HIF-1α mRNA expression or HIF-1α protein degradation, suggesting a direct inhibition of HIF-1 protein synthesis. HIF-1α protein synthesis is regulated by activation of the extracellular signal-regulated kinase-1/2 (ERK1/2). The levels of phosphorylation of ERK1/2, MAPK-interacting protein kinase-1 (MNK1) and eukaryotic initiation factor 4E (eIF4E) were significantly suppressed by the 6α-tigloyloxychaparrinone treatment. The inhibition of this MAPK cascade (HIF-1α upstream elements) is likely responsible for HIF-1α protein inhibition. Different quassinoids are able to interfere with the MAPK pathway. This pathway is a potential target for cancer therapy because it plays a role in the cell acquired growth signals autonomy [52].

Moreover, the mRNA cap-binding protein, eIF4E is overexpressed in a variety of human tumors, and has been associated with cancer progression [53, 54]. Over-expression of eIF4E in experimental models dramatically alters cellular morphology, enhances cell proliferation, and induces cellular transformation, tumorigenesis, and metastasis. In preclinical tumor models, elF4E inhibition repressed tumor growth, and recently, elF4E has been proposed as a new candidate target for anticancer therapeutics [55]. The ability of 6α-tigloyloxychaparrinone to inhibit eIF4E phosphorylation might be the cause of inhibited HIF-1α translation, and thus, anticancer activity. The idea that a single compound can inhibit distinct molecular cascades correlated with tumorigenesis is fascinating, and highlights the need of a more accurate analysis of the broad capabilities of this quassinoid. Among the quassinoids with anti-tumor activities, this compound has a rare tigloyloxy group in position 6. This might be the reason behind the activities exerted by this molecule. This compound could be an important novel candidate for future investigations.

SEMI-SYNTHETIC QUASSINOID ANALOGS

Semi-synthesis plays a major role in the structural modification of natural products, and can lead to the discovery of more active and/or less toxic compounds [56]. Semi-synthetic procedures to modify quassinoid molecules are presently well established [10]. The first investigations on semi-synthetic quassinoids involved dimeric derivatives. Connecting two molecules of bruceantin (**1**) or brusatol (**3**) at the C-3 hydroxy group through malonate, glutarate, adipate and sebacate esters, Lee *et al*. generated bis-esters (**105-110**) active against leukemic cells (P-388) [57]. Brusatol dimers had higher potency as antineoplastic agents than brusatol itself, while brusatol was more active as an anti-inflammatory or differentiating agent.

As previously discussed, the identity of the C-15 ester side chain is a structural feature which was shown to be important for the cytotoxic activity of quassinoids. It has been postulated that oxidation of the C-15 side chain can cause quassinoid inactivation. To circumvent a possible deactivation occurring through this mechanism, trifluoro-methyl groups have been incorporated into the C-15 side chain [58]. The most potent trifluoro-analog was 15-[3′-(trifluoromethyl)butanoyl]bruceolide (**104**), which had a similar potency to bruceantin against a panel of human cancer cell lines. A lipophilic ester side chain at C-15 appeared to be important for both potency and spectrum of response, because it aided the transport across cell membranes. A lipophilic derivative, NBT-272 (**77**), was found to be 2- to 10-fold more potent than the parental compound, bruceantin, in inhibiting the cellular proliferation of a variety of cancer cell lines [8, 59]. Similarly to bruceantin, the derivative also down-regulates the MYC oncogene. The effect of this compound on different types of tumors, in which MYC plays an important role, has been investigated. In particular, it was tested on *in vitro* models of neuroblastoma and medulloblastoma, two childhood cancers of the nervous system frequently characterized by MYC deregulation associated with aggressiveness and poorly differentiated tumors [60-62]. The derivative demonstrated a highly potent anti-proliferative effect. Cells with MYC amplification or over-expression have been found to be more sensitive and prone to apoptosis upon NBT-272 treatment. However, all cell lines tested were strongly affected, suggesting that MYC expression is not the only factor regulating cellular sensitivity to NBT-272 [8]. A more recent study described the wide range of biological activities exerted by the quassinoid derivative NBT-272 on a panel of embryonal tumor cell lines [63]. The inhibition of protein synthesis appeared to be associated with alteration in the protein translation machinery, caused by the inhibition of the phosphorylation of elF4E and its binding protein 4EBP-1. The depletion of MYC protein was associated with cell cycle arrest and occured *via* an indirect mechanism involving the inhibition of two pro-proliferative pathways, i.e. the AKT and the MEK/extracellular signal-regulated kinase (ERK) pathways. Moreover, the compound triggered autophagosome formation and arrested tumor growth in a xenograft model of neuroblastoma.

CONCLUSIONS AND FUTURE PERSPECTIVES

Quassinoids represent a heterogeneous group of molecules with the ability to interfere with different pathways implicated in tumorigenesis. As discussed in the present review, protein synthesis inhibition and cytotoxicity appear to be common characteristics of quassinoids, shared by compounds belonging to different C-type classes. The induction of apoptosis also appears to be a common feature among quassinoids. Several apoptosis-related processes have been found altered upon treatment with members of this class of compounds. Cell death could be related to treatment-induced stress, but on the other hand, the activation of different components of the apoptotic machinery appears to be associated with specific structural characteristics of the individual molecules. For instance, both bruceantin (**1**) and brucein-D (**2**) induce the proteolytic activation of caspase-3, -8 and -9 and proteolytic processing of BID. However, brucein-D induces cell death *via* a complex mechanism involving GSH depletion, oxidative stress and p38-MAPK activation, together with prevention of NF-κB translocation into the nucleus and the consequent inhibition of anti-apoptotic gene expression. It is interesting to note that the quassinoid, brusatol (**3**), induces an opposite effect, promoting NF-κB translocation into the nucleous *via* induction of phosphorylation of IκBα. These diverse effects on the NF-κB pathway may also reflect differences in the cellular models used to study the mechanism of action of the two quassinoids.

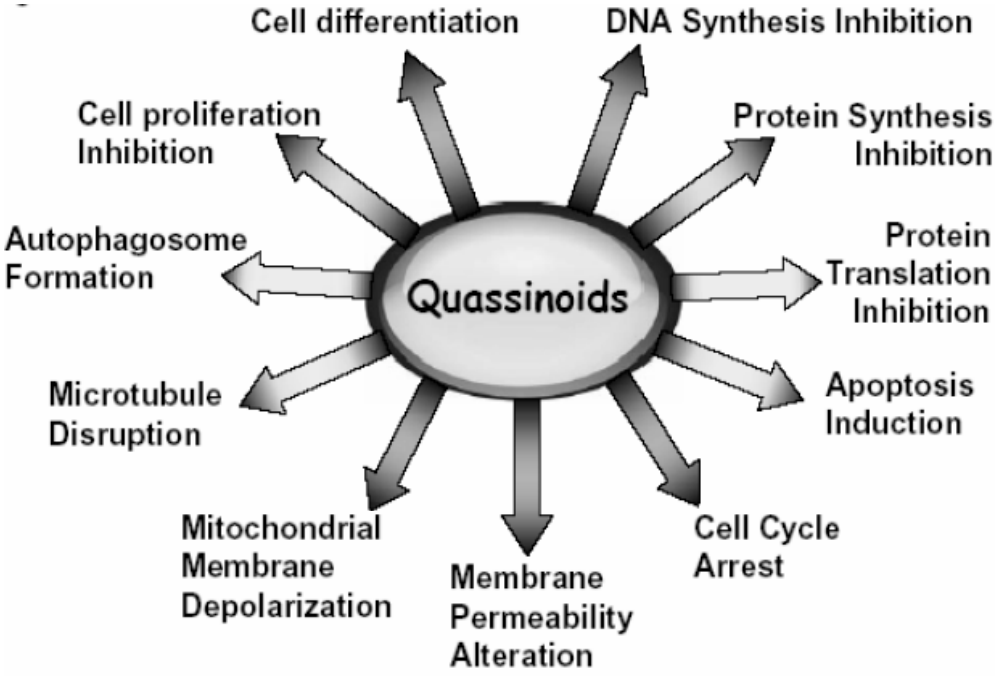

Fig. (2). Cellular processes associated with anticancer activity of quassinoids.

The down-regulation of the MYC oncogene is frequently observed upon quassinoid treatment [8, 16, 25, 27, 45]. Down-regulation of MYC is commonly induced by a subset of C20-type quassinoids [e.g. bruceantin (**1**), brusatol (**3**), NBT-272 (**77**) and AECHL-1 (**93**)]. The MYC family of genes encodes transcription factors that play essential roles in cell proliferation, differentiation, apoptosis and many other cellular processes [64-66]. The deregulation of this oncogene contributes to the genesis of many human cancers, with the development of more aggressive tumors, and is often correlated with poor patient prognosis [67, 68]. Moreover, MYC suppression has been associated with proliferative arrest and tumor regression in a variety of cancer models [69, 70]. Therefore, MYC is an attractive target for cancer treatment [71], and further advances in understanding the mechanism of action of quassinoids could lead to potentially selective derivatives capable of specific inhibition of this oncogene family.

In addition to the induction of apoptosis, cell growth inhibition and MYC reduction, a new mechanism of action among quassinoids has been described for AECHL-1 (**93**), which appears to be a promising microtubule damaging agent. For this reason, we classify the quassinoids as "multi-targeted agents" [72], a class of molecules targeting multiple signaling pathways and cellular processes necessary for the life of a cancer cell (Fig. **2**). The available data have been generated by studying quassinoids in different models, and most of these studies have never been systematically compared. So, it is not possible to exclude that the specific cellular effects, which have been described for one or a few members of the quassinoid family, are more generally shared. However, the broad spectrum of biological effects highlights a need for more accurate and systematic screening of these molecules, and a rational drug design-based study to identify leads for the synthesis or semi-synthesis of new analogs with increased activity, decreased toxicity and/or improved pharmacological profiles for the development of future cancer therapeutics.

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